

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 June 2001 (14.06.2001)

PCT

(10) International Publication Number  
**WO 01/41799 A1**

(51) International Patent Classification<sup>7</sup>: **A61K 39/00,**  
39/12

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(21) International Application Number: PCT/US00/33549

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(22) International Filing Date:

11 December 2000 (11.12.2000)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/172,705 10 December 1999 (10.12.1999) US  
09/641,528 15 August 2000 (15.08.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Published:

- With international search report.
- Before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments.

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: INDUCING CELLULAR IMMUNE RESPONSES TO HUMAN PAPILLOMAVIRUS USING PEPTIDE AND NU-  
CLEIC ACID COMPOSITIONS

(57) Abstract: This invention uses our knowledge of the mechanisms by which antigen is recognized by T cells to identify and  
prepare human papillomavirus (HPV) epitopes, and to develop epitope-based vaccines directed towards HPV. More specifically, this  
application communicates our discovery of pharmaceutical compositions and methods of use in the prevention and treatment of HPV  
infection.

WO 01/41799 A1

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## INDUCING CELLULAR IMMUNE RESPONSES TO HUMAN PAPILLOMAVIRUS USING PEPTIDE AND NUCLEIC ACID COMPOSITIONS

### I. BACKGROUND OF THE INVENTION

10 Human papillomavirus (HPV) is a member of the papillomaviridae, a group of small DNA viruses that infect a variety of higher vertebrates. More than 80 types of HPVs have been identified. Of these, more than 30 can infect the genital tract. Some types, generally types 6 and 11, may cause genital warts, which are typically benign and rarely develop into cancer. Other strains of HPV, "cancer-associated", or "high-risk" types, can more frequently lead to the development of cancer. The primary  
15 mode of transmission of these strains of HPV is through sexual contact.

The main manifestations of the genital warts are cauliflower-like condylomata acuminata that usually involve moist surfaces; keratotic and smooth papular warts, usually on dry surfaces; and subclinical "flat" warts, which are found on any mucosal or cutaneous surface (Handsfield, H., *Am. J. Med.* 102(5A):16-20, 1997). These warts are typically benign but are a source of inter-individual spread of the virus (Ponten, J. & Guo, Z., *Cancer Surv.* 32:201-29, 1998). At least three HPV strains associated with  
20 genital warts have been identified: type 6a (see, e.g., Hofmann, K.J., *et al.*, *Virology* 209(2):506-518, 1995), type 6b (see, e.g., Hofmann *et al.*, *supra*) and type 11 (see, e.g., Dartmann, K. *et al.*, *Virology* 151(1):124-130, 1986).

Cancer-associated HPVs have been linked with cancer in both men and women; they  
25 include, but are not limited to, HPV-16, HPV-18, HPV-31, HPV-45, HPV-33 and HPV-56. Other HPV strains, including types 6 and 11 as well as others, e.g., HPV-5 and HPV-8, are less frequently associated with cancer. The high risk types are typically associated with the development of cervical carcinoma and premalignant lesions of the cervix in women, but are also associated with similar malignant and  
pre-malignant lesions at other anatomic sites within the lower genital or anogenital tract. These lesions  
30 include neoplasia of the vagina, vulva, perineum, the penis, and the anus. HPV infection has also been associated with respiratory tract papillomas, and rarely, cancer, as well as abnormal growth or neoplasia in other epithelial tissues. See, e.g. *VIROLOGY*, 2<sup>ND</sup> ED, Fields *et al.*, Eds. Raven Press, New York, 1990, Chapters 58 and 59, for a review of HPV association with cancer.

The HPV genome consists of three functional regions, the early region, the late region,  
35 and the "long control region". The early region gene products control viral replication, transcription and cellular transformation. They include the HPV E1 and E2 proteins, which play a role in HPV DNA replication, and the E6 and E7 oncoproteins, which are involved in the control of cellular proliferation. The late region include the genes that encode the structural proteins L1 and L2, which are the major and minor capsid proteins, respectively. The "long control region" contains such sequences as enhancer and promoter  
40 regulatory regions.

HPV expresses different proteins at different stages of the infection, for example early, as well as late, proteins. Even in latent infections, however, early proteins are often expressed and are therefore useful targets for vaccine-based therapies. For example, high-grade dysplasia and cervical squamous cell carcinoma continue to express E6 and E7, which therefore can be targeted to treat disease at both early and late stages of infection.

Treatment for HPV infection is often unsatisfactory because of persistence of virus after treatment and recurrence of clinically apparent disease is common. The treatment may require frequent visits to clinics and is not directed at elimination of the virus but at clearing warts. Because of persistence of virus after treatment, recurrence of clinically apparent disease is common.

Thus, a need exists for an efficacious vaccine to both prevent and treat HPV infection and to treat cancer that is associated with HPV infection. Effective HPV vaccines would be a significant advance in the control of sexually transmissible infections and could also protect against clinical disease, particularly cancers such as cervical cancer. (see, e.g., Rowen, P. & Lacey, C., *Dermatologic Clinics* 16(4):835-838, 1998).

Virus-specific, human leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTL) are known to play a major role in the prevention and clearance of virus infections *in vivo* (Oldstone *et al.*, *Nature* 321:239, 1989; Jamieson *et al.*, *J. Virol.* 61:3930, 1987; Yap *et al.*, *Nature* 273:238, 1978; Lukacher *et al.*, *J. Exp. Med.* 160:814, 1994; McMichael *et al.*, *N. Engl. J. Med.* 309:13, 1983; Sethi *et al.*, *J. Gen. Virol.* 64:443, 1983; Watari *et al.*, *J. Exp. Med.* 165:459, 1987; Yasukawa *et al.*, *J. Immunol.* 143:2051, 1989; Tigges *et al.*, *J. Virol.* 66:1622, 1993; Reddenhase *et al.*, *J. Virol.* 55:263, 1985; Quinnan *et al.*, *N. Engl. J. Med.* 307:6, 1982). HLA class I molecules are expressed on the surface of almost all nucleated cells. Following intracellular processing of antigens, epitopes from the antigens are presented as a complex with the HLA class I molecules on the surface of such cells. CTL recognize the peptide-HLA class I complex, which then results in the destruction of the cell bearing the HLA-peptide complex directly by the CTL and/or via the activation of non-destructive mechanisms e.g., the production of interferon, that inhibit viral replication.

Virus-specific T helper lymphocytes are also known to be critical for maintaining effective immunity in chronic viral infections. Historically, HTL responses were viewed as primarily supporting the expansion of specific CTL and B cell populations; however, more recent data indicate that HTL may directly contribute to the control of virus replication. For example, a decline in CD4<sup>+</sup> T cells and a corresponding loss in HTL function characterize infection with HIV (Lane *et al.*, *New Engl. J. Med.* 313:79, 1985). Furthermore, studies in HIV infected patients have also shown that there is an inverse relationship between virus-specific HTL responses and viral load, suggesting that HTL plays a role in viremia (see, e.g., Rosenberg *et al.*, *Science* 278:1447, 1997).

The development of vaccines with prophylactic and therapeutic efficacy against HPV is ongoing. Early vaccine development was hampered by the inability to culture HPV. With the introduction of cloning techniques and protein expression, however, some attempts have been made to stimulate humoral and CTL response to HPV (See, e.g., Rowen, P. & Lacey, C., *Dermatologic Clinics* 16(4):835-838 (1998)). Studies to date, however, have been inconclusive.

Activation of T helper cells and cytotoxic lymphocytes (CTLs) in the development of vaccines has also been analyzed. Lehtinen, M., *et al.* for instance, has shown that some peptides from the E2 protein of HPV type 16 activate T helper cells and CTLs (*Biochem. Biophys. Res. Commun.* 209(2):541-6 (1995). Similarly, Tarpey *et al.* has shown that some peptides from HPV type 11 E7 protein can stimulate human HPV-specific CTLs *in vitro* (*Immunology* 81:222-227 (1994)) and Borysiewicz *et al.* have reported a recombinant vaccinia virus expressing HPV 16 and HPV 17 E6 and E7 that stimulated CTL responses in at least one patient (*Lancet* 347:1347-1357, 1996).

The epitope approach, as we have described, allows the incorporation of various antibody, CTL and HTL epitopes, from various proteins, in a single vaccine composition. Such a composition may simultaneously target multiple dominant and subdominant epitopes and thereby be used to achieve effective immunization in a diverse population.

The information provided in this section is intended to disclose the presently understood state of the art as of the filing date of the present application. Information is included in this section which was generated subsequent to the priority date of this application. Accordingly, information in this section is not intended, in any way, to delineate the priority date for the invention.

## II. SUMMARY OF THE INVENTION

This invention applies our knowledge of the mechanisms by which antigen is recognized by T cells, for example, to develop epitope-based vaccines directed towards HPV. More specifically, this application communicates our discovery of specific epitope pharmaceutical compositions and methods of use in the prevention and treatment of HPV infection.

Upon development of appropriate technology, the use of epitope-based vaccines has several advantages over current vaccines, particularly when compared to the use of whole antigens in vaccine compositions. There is evidence that the immune response to whole antigens is directed largely toward variable regions of the antigen, allowing for immune escape due to mutations. The epitopes for inclusion in an epitope-based vaccine may be selected from conserved regions of viral or tumor-associated antigens, which thereby reduces the likelihood of escape mutants. Furthermore, immunosuppressive epitopes that may be present in whole antigens can be avoided with the use of epitope-based vaccines.

An additional advantage of an epitope-based vaccine approach is the ability to combine selected epitopes (CTL and HTL), and further, to modify the composition of the epitopes, achieving, for example, enhanced immunogenicity. Accordingly, the immune response can be modulated, as appropriate, for the target disease. Similar engineering of the response is not possible with traditional approaches.

Another major benefit of epitope-based immune-stimulating vaccines is their safety. The possible pathological side effects caused by infectious agents or whole protein antigens, which might have their own intrinsic biological activity, is eliminated.

An epitope-based vaccine also provides the ability to direct and focus an immune response to multiple selected antigens from the same pathogen. Thus, patient-by-patient variability in the immune response to a particular pathogen may be alleviated by inclusion of epitopes from multiple antigens from the pathogen in a vaccine composition. In the case of HPV, epitopes derived from multiple strains may also be included. A "pathogen" may be an infectious agent or a tumor associated molecule.

One of the most formidable obstacles to the development of broadly efficacious epitope-based immunotherapeutics, however, has been the extreme polymorphism of HLA molecules. To date, effective non-genetically biased coverage of a population has been a task of considerable complexity; such coverage has required that epitopes be used that are specific for HLA molecules corresponding to each individual HLA allele. Impractically large numbers of epitopes would therefore have to be used in order to cover ethnically diverse populations. Thus, there has existed a need for peptide epitopes that are bound by multiple HLA antigen molecules for use in epitope-based vaccines. The greater the number of HLA antigen molecules bound, the greater the breadth of population coverage by the vaccine.

Furthermore, as described herein in greater detail, a need has existed to modulate peptide binding properties, *e.g.*, so that peptides that are able to bind to multiple HLA antigens do so with an affinity that will stimulate an immune response. Identification of epitopes restricted by more than one HLA allele at an affinity that correlates with immunogenicity is important to provide thorough population coverage, and to allow the elicitation of responses of sufficient vigor to prevent or clear an infection in a diverse segment of the population. Such a response can also target a broad array of epitopes. The technology disclosed herein provides for such favored immune responses.

In a preferred embodiment, epitopes for inclusion in vaccine compositions of the invention are selected by a process whereby protein sequences of known antigens are evaluated for the presence of motif or supermotif-bearing epitopes. Peptides corresponding to a motif- or supermotif-bearing epitope are then synthesized and tested for the ability to bind to the HLA molecule that recognizes the selected motif. Those peptides that bind at an intermediate or high affinity *i.e.*, an  $IC_{50}$  (or a  $K_D$  value) of 500 nM or less for HLA class I molecules or an  $IC_{50}$  of 1000 nM or less for HLA class II molecules, are further evaluated for their ability to induce a CTL or HTL response. Immunogenic peptide epitopes are selected for inclusion in vaccine compositions.

Supermotif-bearing peptides may additionally be tested for the ability to bind to multiple alleles within the HLA supertype family. Moreover, peptide epitopes may be analogued to modify binding affinity and/or the ability to bind to multiple alleles within an HLA supertype.

The invention also includes embodiments comprising methods for monitoring or evaluating an immune response to HPV in a patient having a known HLA-type. Such methods comprise incubating a T lymphocyte sample from the patient with a peptide composition comprising an HPV epitope that has an amino acid sequence described in Tables VII to Table XX which binds the product of at least one HLA allele present in the patient, and detecting for the presence of a T lymphocyte that binds to the peptide. A CTL peptide epitope may, for example, be used as a component of a tetrameric complex for this type of analysis.

An alternative modality for defining the peptide epitopes in accordance with the invention is to recite the physical properties, such as length; primary structure; or charge, which are correlated with binding to a particular allele-specific HLA molecule or group of allele-specific HLA molecules. A further modality for defining peptide epitopes is to recite the physical properties of an HLA binding pocket, or properties shared by several allele-specific HLA binding pockets (*e.g.* pocket configuration and charge distribution) and reciting that the peptide epitope fits and binds to the pocket or pockets.

As will be apparent from the discussion below, other methods and embodiments are also contemplated. Further, novel synthetic peptides produced by any of the methods described herein are also part of the invention.

### 5 III. DETAILED DESCRIPTION OF THE INVENTION

The peptides and corresponding nucleic acid compositions of the present invention are useful for stimulating an immune response to HPV by stimulating the production of CTL or HTL responses. The peptide epitopes, which are derived directly or indirectly from native HPV protein amino acid sequences, are able to bind to HLA molecules and stimulate an immune response to HPV. The complete  
10 sequence of the HPV proteins to be analyzed can be obtained from Genbank. Epitopes and analogs thereof can also be readily determined from sequence information that may subsequently be discovered for heretofore unknown variants of HPV, as will be clear from the disclosure provided below.

The epitopes of the invention have been identified in a number of ways, as will be discussed below. Also discussed in greater detail is that analog peptides have been derived and the binding  
15 activity for HLA molecules modulated by modifying specific amino acid residues to create peptide analogs exhibiting altered immunogenicity. Further, the present invention provides compositions and combinations of compositions that enable epitope-based vaccines that are capable of interacting with HLA molecules encoded by various genetic alleles to provide broader population coverage than prior vaccines.

#### 20 III.A. Definitions

The invention can be better understood with reference to the following definitions, which are listed alphabetically:

A "computer" or "computer system" generally includes: a processor; at least one information storage/retrieval apparatus such as, for example, a hard drive, a disk drive or a tape drive; at  
25 least one input apparatus such as, for example, a keyboard, a mouse, a touch screen, or a microphone; and display structure. Additionally, the computer may include a communication channel in communication with a network. Such a computer may include more or less than what is listed above.

A "construct" as used herein generally denotes a composition that does not occur in nature. A construct can be produced by synthetic technologies, *e.g.*, recombinant DNA preparation and  
30 expression or chemical synthetic techniques for nucleic or amino acids. A construct can also be produced by the addition or affiliation of one material with another such that the result is not found in nature in that form.

"Cross-reactive binding" indicates that a peptide is bound by more than one HLA molecule; a synonym is degenerate binding.

A "cryptic epitope" elicits a response by immunization with an isolated peptide, but the  
35 response is not cross-reactive *in vitro* when intact whole protein which comprises the epitope is used as an antigen.

A "dominant epitope" is an epitope that induces an immune response upon immunization with a whole native antigen (see, *e.g.*, Sercarz, *et al.*, *Annu. Rev. Immunol.* 11:729-766, 1993). Such a  
40 response is cross-reactive *in vitro* with an isolated peptide epitope.

With regard to a particular amino acid sequence, an "epitope" is a set of amino acid residues which is involved in recognition by a particular immunoglobulin, or in the context of T cells, those residues necessary for recognition by T cell receptor proteins and/or Major Histocompatibility Complex (MHC) receptors. In an immune system setting, *in vivo* or *in vitro*, an epitope is the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or HLA molecule. Throughout this disclosure epitope and peptide are often used interchangeably. It is to be appreciated, however, that isolated or purified protein or peptide molecules larger than and comprising an epitope of the invention are still within the bounds of the invention.

"Human Leukocyte Antigen" or "HLA" is a human class I or class II Major Histocompatibility Complex (MHC) protein (*see, e.g., Stites, et al., IMMUNOLOGY*, 8<sup>TH</sup> ED., Lange Publishing, Los Altos, CA (1994).

An "HLA supertype or family", as used herein, describes sets of HLA molecules grouped on the basis of shared peptide-binding specificities. HLA class I molecules that share somewhat similar binding affinity for peptides bearing certain amino acid motifs are grouped into HLA supertypes. The terms HLA superfamily, HLA supertype family, HLA family, and HLA xx-like molecules (where xx denotes a particular HLA type), are synonyms.

Throughout this disclosure, results are expressed in terms of "IC<sub>50</sub>'s." IC<sub>50</sub> is the concentration of peptide in a binding assay at which 50% inhibition of binding of a reference peptide is observed. Given the conditions in which the assays are run (*i.e., limiting* HLA proteins and labeled peptide concentrations), these values approximate K<sub>D</sub> values. Assays for determining binding are described in detail, *e.g., in* PCT publications WO 94/20127 and WO 94/03205. It should be noted that IC<sub>50</sub> values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (*e.g., HLA preparation, etc.*). For example, excessive concentrations of HLA molecules will increase the apparent measured IC<sub>50</sub> of a given ligand.

Alternatively, binding is expressed relative to a reference peptide. Although as a particular assay becomes more, or less, sensitive, the IC<sub>50</sub>'s of the peptides tested may change somewhat, the binding relative to the reference peptide will not significantly change. For example, in an assay run under conditions such that the IC<sub>50</sub> of the reference peptide increases 10-fold, the IC<sub>50</sub> values of the test peptides will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder is generally based on its IC<sub>50</sub>, relative to the IC<sub>50</sub> of a standard peptide.

Binding may also be determined using other assay systems including those using: live cells (*e.g., Ceppellini et al., Nature* 339:392, 1989; Christnick *et al., Nature* 352:67, 1991; Busch *et al., Int. Immunol.* 2:443, 1990; Hill *et al., J. Immunol.* 147:189, 1991; del Guercio *et al., J. Immunol.* 154:685, 1995), cell free systems using detergent lysates (*e.g., Cerundolo et al., J. Immunol.* 21:2069, 1991), immobilized purified MHC (*e.g., Hill et al., J. Immunol.* 152, 2890, 1994; Marshall *et al., J. Immunol.* 152:4946, 1994), ELISA systems (*e.g., Reay et al., EMBO J.* 11:2829, 1992), surface plasmon resonance (*e.g., Khilko et al., J. Biol. Chem.* 268:15425, 1993); high flux soluble phase assays (Hammer *et al., J. Exp. Med.* 180:2353, 1994), and measurement of class I MHC stabilization or assembly (*e.g., Ljunggren et al.,*

*Nature* 346:476, 1990; Schumacher *et al.*, *Cell* 62:563, 1990; Townsend *et al.*, *Cell* 62:285, 1990; Parker *et al.*, *J. Immunol.* 149:1896, 1992).

As used herein, "high affinity" with respect to HLA class I molecules is defined as binding with an  $IC_{50}$  or  $K_D$  value, of 50 nM or less; "intermediate affinity" is binding with an  $IC_{50}$  or  $K_D$  value of between about 50 and about 500 nM. "High affinity" with respect to binding to HLA class II molecules is defined as binding with an  $IC_{50}$  or  $K_D$  value of 100 nM or less; "intermediate affinity" is binding with an  $IC_{50}$  or  $K_D$  value of between about 100 and about 1000 nM.

The terms "identical" or percent "identity," in the context of two or more peptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using a sequence comparison algorithm or by manual alignment and visual inspection.

An "immunogenic peptide" or "peptide epitope" is a peptide that comprises an allele-specific motif or supermotif such that the peptide will bind an HLA molecule and induce a CTL and/or HTL response. Thus, immunogenic peptides of the invention are capable of binding to an appropriate HLA molecule and thereafter inducing a cytotoxic T cell response, or a helper T cell response, to the antigen from which the immunogenic peptide is derived.

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany the material as it is found in its native state. Thus, isolated peptides in accordance with the invention preferably do not contain materials normally associated with the peptides in their *in situ* environment.

"Link" or "join" refers to any method known in the art for functionally connecting peptides, including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, and electrostatic bonding.

"Major Histocompatibility Complex" or "MHC" is a cluster of genes that plays a role in control of the cellular interactions responsible for physiologic immune responses. In humans, the MHC complex is also known as the HLA complex. For a detailed description of the MHC and HLA complexes, see, Paul, *FUNDAMENTAL IMMUNOLOGY*, 3<sup>RD</sup> ED., Raven Press, New York, 1993.

The term "motif" refers to the pattern of residues in a peptide of defined length, usually a peptide of from about 8 to about 13 amino acids for a class I HLA motif and from about 6 to about 25 amino acids for a class II HLA motif, which is recognized by a particular HLA molecule. Peptide motifs are typically different for each protein encoded by each human HLA allele and differ in the pattern of the primary and secondary anchor residues.

A "negative binding residue" or "deleterious residue" is an amino acid which, if present at certain positions (typically not primary anchor positions) in a peptide epitope, results in decreased binding affinity of the peptide for the peptide's corresponding HLA molecule.

A "non-native" sequence or "construct" refers to a sequence that is not found in nature, i.e., is "non-naturally occurring". Such sequences include, e.g., peptides that are lipidated or otherwise modified, and polyepitopic compositions that contain epitopes that are not contiguous in a native protein sequence.



The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other, typically by peptide bonds between the  $\alpha$ -amino and carboxyl groups of adjacent amino acids. The preferred CTL-inducing peptides of the invention are 13 residues or less in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues. The preferred HTL-inducing oligopeptides are less than about 50 residues in length and usually consist of between about 6 and about 30 residues, more usually between about 12 and 25, and often between about 15 and 20 residues.

It is to be appreciated that protein or peptide molecules that comprise an epitope of the invention as well as additional amino acid(s) are within the bounds of the invention. In certain embodiments, there is a limitation on the length of a peptide of the invention which is not otherwise a construct as defined herein. An embodiment that is length-limited occurs when the protein/peptide comprising an epitope of the invention comprises a region (*i.e.*, a contiguous series of amino acids) having 100% identity with a native sequence. In order to avoid a recited definition of epitope from reading, *e.g.*, on whole natural molecules, the length of any region that has 100% identity with a native peptide sequence is limited. Thus, for a peptide comprising an epitope of the invention and a region with 100% identity with a native peptide sequence (and which is not otherwise a construct), the region with 100% identity to a native sequence generally has a length of: less than or equal to 600 amino acids, often less than or equal to 500 amino acids, often less than or equal to 400 amino acids, often less than or equal to 250 amino acids, often less than or equal to 100 amino acids, often less than or equal to 85 amino acids, often less than or equal to 75 amino acids, often less than or equal to 65 amino acids, and often less than or equal to 50 amino acids. In certain embodiments, an "epitope" of the invention which is not a construct is comprised by a peptide having a region with less than 51 amino acids that has 100% identity to a native peptide sequence, in any increment of (50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5) down to 5 amino acids.

Certain peptide or protein sequences longer than 600 amino acids are within the scope of the invention. Such longer sequences are within the scope of the invention so long as they do not comprise any contiguous sequence of more than 600 amino acids that have 100% identity with a native peptide sequence, or if longer than 600 amino acids, they are a construct. For any peptide that has five contiguous residues or less that correspond to a native sequence, there is no limitation on the maximal length of that peptide in order to fall within the scope of the invention. It is presently preferred that a CTL epitope of the invention be less than 600 residues long in any increment down to eight amino acid residues.

"Pharmaceutically acceptable" refers to a non-toxic, inert, and/or physiologically compatible composition.

A "pharmaceutical excipient" comprises a material such as an adjuvant, a carrier, pH-adjusting and buffering agents, tonicity adjusting agents, wetting agents, preservative, and the like.

A "primary anchor residue" is an amino acid at a specific position along a peptide sequence which is understood to provide a contact point between the immunogenic peptide and the HLA molecule. One to three, usually two, primary anchor residues within a peptide of defined length generally defines a "motif" for an immunogenic peptide. These residues are understood to fit in close contact with peptide binding grooves of an HLA molecule, with their side chains buried in specific pockets of the

binding grooves themselves. In one embodiment, for example, the primary anchor residues are located at position 2 (from the amino terminal position) and at the carboxyl terminal position of a 9-residue peptide epitope in accordance with the invention. The primary anchor positions for each motif and supermotif are set forth in Table 1. For example, analog peptides can be created by altering the presence or absence of particular residues in these primary anchor positions. Such analogs are used to modulate the binding affinity of a peptide comprising a particular motif or supermotif.

"Promiscuous recognition" is where a distinct peptide is recognized by the same T cell clone in the context of various HLA molecules. Promiscuous recognition or binding is synonymous with cross-reactive binding.

A "protective immune response" or "therapeutic immune response" refers to a CTL and/or an HTL response to an antigen derived from an infectious agent or a tumor antigen, which prevents or at least partially arrests disease symptoms or progression. The immune response may also include an antibody response which has been facilitated by the stimulation of helper T cells.

The term "residue" refers to an amino acid or amino acid mimetic incorporated into an oligopeptide by an amide bond or amide bond mimetic.

A "secondary anchor residue" is an amino acid at a position other than a primary anchor position in a peptide which may influence peptide binding. A secondary anchor residue occurs at a significantly higher frequency amongst bound peptides than would be expected by random distribution of amino acids at one position. The secondary anchor residues are said to occur at "secondary anchor positions." A secondary anchor residue can be identified as a residue which is present at a higher frequency among high or intermediate affinity binding peptides, or a residue otherwise associated with high or intermediate affinity binding. For example, analog peptides can be created by altering the presence or absence of particular residues in these secondary anchor positions. Such analogs are used to finely modulate the binding affinity of a peptide comprising a particular motif or supermotif.

A "subdominant epitope" is an epitope which evokes little or no response upon immunization with whole antigens which comprise the epitope, but for which a response can be obtained by immunization with an isolated peptide, and this response (unlike the case of cryptic epitopes) is detected when whole protein is used to recall the response *in vitro* or *in vivo*.

A "supermotif" is a peptide binding specificity shared by HLA molecules encoded by two or more HLA alleles. Preferably, a supermotif-bearing peptide is recognized with high or intermediate affinity (as defined herein) by two or more HLA antigens.

"Synthetic peptide" refers to a peptide that is man-made using such methods as chemical synthesis or recombinant DNA technology.

As used herein, a "vaccine" is a composition that contains one or more peptides of the invention. There are numerous embodiments of vaccines in accordance with the invention, such as by a cocktail of one or more peptides; one or more epitopes of the invention comprised by a polypeptidic peptide; or nucleic acids that encode such peptides or polypeptides, *e.g.*, a minigene that encodes a polypeptidic peptide. The "one or more peptides" can include any whole unit integer from 1-150, *e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125,

130, 135, 140, 145, or 150 or more peptides of the invention. The peptides or polypeptides can optionally be modified, such as by lipidation, addition of targeting or other sequences. HLA class I-binding peptides of the invention can be admixed with, or linked to, HLA class II-binding peptides, to facilitate activation of both cytotoxic T lymphocytes and helper T lymphocytes. Vaccines can also comprise peptide-pulsed antigen presenting cells, e.g., dendritic cells.

The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. When amino acid residue positions are referred to in a peptide epitope they are numbered in an amino to carboxyl direction with position one being the position closest to the amino terminal end of the epitope, or the peptide or protein of which it may be a part. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G. The amino acid sequences of peptides set forth herein are generally designated using the standard single letter symbol. (A, Alanine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; and Y, Tyrosine.) In addition to these symbols, "B" in the single letter abbreviations used herein designates  $\alpha$ -amino butyric acid.

### III.B. Stimulation of CTL and HTL responses

The mechanism by which T cells recognize antigens has been delineated during the past ten years. Based on our understanding of the immune system we have developed efficacious peptide epitope vaccine compositions that can induce a therapeutic or prophylactic immune response to HPV in a broad population. For an understanding of the value and efficacy of the claimed compositions, a brief review of immunology-related technology is provided.

A complex of an HLA molecule and a peptidic antigen acts as the ligand recognized by HLA-restricted T cells (Buus, S. *et al.*, *Cell* 47:1071, 1986; Babbitt, B. P. *et al.*, *Nature* 317:359, 1985; Townsend, A. and Bodmer, H., *Annu. Rev. Immunol.* 7:601, 1989; Germain, R. N., *Annu. Rev. Immunol.* 11:403, 1993). Through the study of single amino acid substituted antigen analogs and the sequencing of endogenously bound, naturally processed peptides, critical residues that correspond to motifs required for specific binding to HLA antigen molecules have been identified and are described herein and are set forth in Tables I, II, and III (see also, e.g., Southwood, *et al.*, *J. Immunol.* 160:3363, 1998; Rammensee, *et al.*, *Immunogenetics* 41:178, 1995; Rammensee *et al.*, SYFPEITHI, access via web at : <http://134.2.96.221/scripts.hlaserver.dll/home.htm>; Sette, A. and Sidney, J. *Curr. Opin. Immunol.* 10:478, 1998; Engelhard, V. H., *Curr. Opin. Immunol.* 6:13, 1994; Sette, A. and Grey, H. M., *Curr. Opin. Immunol.* 4:79, 1992; Sinigaglia, F. and Hammer, J. *Curr. Biol.* 6:52, 1994; Ruppert *et al.*, *Cell* 74:929-937, 1993;

Kondo *et al.*, *J. Immunol.* 155:4307-4312, 1995; Sidney *et al.*, *J. Immunol.* 157:3480-3490, 1996; Sidney *et al.*, *Human Immunol.* 45:79-93, 1996; Sette, A. and Sidney, J. *Immunogenetics* 1999 Nov;50(3-4):201-12, Review).

Furthermore, x-ray crystallographic analysis of HLA-peptide complexes has revealed  
 5 pockets within the peptide binding cleft of HLA molecules which accommodate, in an allele-specific mode, residues borne by peptide ligands; these residues in turn determine the HLA binding capacity of the peptides in which they are present. (See, e.g., Madden, D.R. *Annu. Rev. Immunol.* 13:587, 1995; Smith, *et al.*, *Immunity* 4:203, 1996; Fremont *et al.*, *Immunity* 8:305, 1998; Stern *et al.*, *Structure* 2:245, 1994; Jones, E.Y. *Curr. Opin. Immunol.* 9:75, 1997; Brown, J. H. *et al.*, *Nature* 364:33, 1993; Guo, H. C. *et al.*, *Proc.*  
 10 *Natl. Acad. Sci. USA* 90:8053, 1993; Guo, H. C. *et al.*, *Nature* 360:364, 1992; Silver, M. L. *et al.*, *Nature* 360:367, 1992; Matsumura, M. *et al.*, *Science* 257:927, 1992; Madden *et al.*, *Cell* 70:1035, 1992; Fremont, D. H. *et al.*, *Science* 257:919, 1992; Saper, M. A., Bjorkman, P. J. and Wiley, D. C., *J. Mol. Biol.* 219:277, 1991.)

Accordingly, the definition of class I and class II allele-specific HLA binding motifs, or  
 15 class I or class II supermotifs allows identification of regions within a protein that have the potential of binding particular HLA antigen(s).

The present inventors have found that the correlation of binding affinity with immunogenicity, which is disclosed herein, is an important factor to be considered when evaluating candidate peptides. Thus, by a combination of motif searches and HLA-peptide binding assays, candidates  
 20 for epitope-based vaccines have been identified. After determining their binding affinity, additional confirmatory work can be performed to select, amongst these vaccine candidates, epitopes with preferred characteristics in terms of population coverage, antigenicity, and immunogenicity.

Various strategies can be utilized to evaluate immunogenicity, including:

1) Evaluation of primary T cell cultures from normal individuals (see, e.g., Wentworth, P. A. *et al.*, *Mol. Immunol.* 32:603, 1995; Celis, E. *et al.*, *Proc. Natl. Acad. Sci. USA* 91:2105, 1994; Tsai, V. *et al.*, *J. Immunol.* 158:1796, 1997; Kawashima, I. *et al.*, *Human Immunol.* 59:1, 1998); This procedure involves the stimulation of peripheral blood lymphocytes (PBL) from normal subjects with a test peptide in the presence of antigen presenting cells *in vitro* over a period of several weeks. T cells specific for the peptide become activated during this time and are detected using, e.g., a lymphokine- or <sup>51</sup>Cr-release assay  
 30 involving peptide sensitized target cells.

2) Immunization of HLA transgenic mice (see, e.g., Wentworth, P. A. *et al.*, *J. Immunol.* 26:97, 1996; Wentworth, P. A. *et al.*, *Int. Immunol.* 8:651, 1996; Alexander, J. *et al.*, *J. Immunol.* 159:4753, 1997); In this method, peptides in incomplete Freund's adjuvant are administered subcutaneously to HLA transgenic mice. Several weeks following immunization, splenocytes are removed and cultured *in vitro* in  
 35 the presence of test peptide for approximately one week. Peptide-specific T cells are detected using, e.g., a <sup>51</sup>Cr-release assay involving peptide sensitized target cells and target cells expressing endogenously generated antigen.

3) Demonstration of recall T cell responses from immune individuals who have effectively been vaccinated, recovered from infection, and/or from chronically infected patients (see, e.g.,  
 40 Rehmann, B. *et al.*, *J. Exp. Med.* 181:1047, 1995; Doolan, D. L. *et al.*, *Immunity* 7:97, 1997; Bertoni, R.

*et al.*, *J. Clin. Invest.* 100:503, 1997; Threlkeld, S. C. *et al.*, *J. Immunol.* 159:1648, 1997; Diepolder, H. M. *et al.*, *J. Virol.* 71:6011, 1997); In applying this strategy, recall responses are detected by culturing PBL from subjects that have been naturally exposed to the antigen, for instance through infection, and thus have generated an immune response "naturally", or from patients who were vaccinated against the infection.

- 5 PBL from subjects are cultured *in vitro* for 1-2 weeks in the presence of test peptide plus antigen presenting cells (APC) to allow activation of "memory" T cells, as compared to "naive" T cells. At the end of the culture period, T cell activity is detected using assays for T cell activity including  $^{51}\text{Cr}$  release involving peptide-sensitized targets, T cell proliferation, or lymphokine release.

10 The following describes the peptide epitopes and corresponding nucleic acids of the invention.

### III.C. Binding Affinity of Peptide Epitopes for HLA Molecules

As indicated herein, the large degree of HLA polymorphism is an important factor to be taken into account with the epitope-based approach to vaccine development. To address this factor, epitope  
15 selection encompassing identification of peptides capable of binding at high or intermediate affinity to multiple HLA molecules is preferably utilized, most preferably these epitopes bind at high or intermediate affinity to two or more allele-specific HLA molecules.

CTL-inducing peptides of interest for vaccine compositions preferably include those that have an  $\text{IC}_{50}$  or binding affinity value for class I HLA molecules of 500 nM or better (*i.e.*, the value is  $\leq 500$   
20 nM). HTL-inducing peptides preferably include those that have an  $\text{IC}_{50}$  or binding affinity value for class II HLA molecules of 1000 nM or better, (*i.e.*, the value is  $\leq 1,000$  nM). For example, peptide binding is assessed by testing the capacity of a candidate peptide to bind to a purified HLA molecule *in vitro*. Peptides exhibiting high or intermediate affinity are then considered for further analysis. Selected peptides are tested on other members of the supertype family. In preferred embodiments, peptides that exhibit cross-  
25 reactive binding are then used in cellular screening analyses or vaccines.

As disclosed herein, higher HLA binding affinity is correlated with greater immunogenicity. Greater immunogenicity can be manifested in several different ways. Immunogenicity corresponds to whether an immune response is elicited at all, and to the vigor of any particular response, as well as to the extent of a population in which a response is elicited. For example, a peptide might elicit an  
30 immune response in a diverse array of the population, yet in no instance produce a vigorous response. In accordance with these principles, close to 90% of high binding peptides have been found to be immunogenic, as contrasted with about 50% of the peptides which bind with intermediate affinity. Moreover, higher binding affinity peptides lead to more vigorous immunogenic responses. As a result, less peptide is required to elicit a similar biological effect if a high affinity binding peptide is used. Thus, in  
35 preferred embodiments of the invention, high affinity binding epitopes are particularly useful.

The relationship between binding affinity for HLA class I molecules and immunogenicity of discrete peptide epitopes on bound antigens has been determined for the first time in the art by the present inventors. The correlation between binding affinity and immunogenicity was analyzed in two different experimental approaches (*see, e.g.*, Sette, *et al.*, *J. Immunol.* 153:5586-5592, 1994). In the first  
40 approach, the immunogenicity of potential epitopes ranging in HLA binding affinity over a 10,000-fold

range was analyzed in HLA-A\*0201 transgenic mice. In the second approach, the antigenicity of approximately 100 different hepatitis B virus (HBV)-derived potential epitopes, all carrying A\*0201 binding motifs, was assessed by using PBL from acute hepatitis patients. Pursuant to these approaches, it was determined that an affinity threshold value of approximately 500 nM (preferably 50 nM or less) determines the capacity of a peptide epitope to elicit a CTL response. These data are true for class I binding affinity measurements for naturally processed peptides and for synthesized T cell epitopes. These data also indicate the important role of determinant selection in the shaping of T cell responses (*see, e.g., Schaeffer et al. Proc. Natl. Acad. Sci. USA* 86:4649-4653, 1989).

An affinity threshold associated with immunogenicity in the context of HLA class II DR molecules has also been delineated (*see, e.g., Southwood et al. J. Immunology* 160:3363-3373, 1998, and co-pending U.S.S.N. 09/009,953 filed 1/21/98). In order to define a biologically significant threshold of DR binding affinity, a database of the binding affinities of 32 DR-restricted epitopes for their restricting element (*i.e.*, the HLA molecule that binds the motif) was compiled. In approximately half of the cases (15 of 32 epitopes), DR restriction was associated with high binding affinities, *i.e.* binding affinity values of 100 nM or less. In the other half of the cases (16 of 32), DR restriction was associated with intermediate affinity (binding affinity values in the 100-1000 nM range). In only one of 32 cases was DR restriction associated with an IC<sub>50</sub> of 1000 nM or greater. Thus, 1000 nM can be defined as an affinity threshold associated with immunogenicity in the context of DR molecules.

In the case of tumor-associated antigens (TAAs), many CTL peptide epitopes that have been shown to induce CTL that lyse peptide-pulsed target cells and tumor cell targets endogenously expressing the epitope exhibit binding affinity or IC<sub>50</sub> values of 200 nM or less. In a study that evaluated the association of binding affinity and immunogenicity of a small set of such TAA epitopes, 100% (10/10) of the high binders, *i.e.*, peptide epitopes binding at an affinity of 50 nM or less, were immunogenic and 80% (8/10) of them elicited CTLs that specifically recognized tumor cells. In the 51 to 200 nM range, very similar figures were obtained. With respect to analog peptides, CTL inductions positive for wildtype peptide and tumor cells were noted for 86% (6/7) and 71% (5/7) of the peptides, respectively. In the 201-500 nM range, most peptides (4/5 wildtype) were positive for induction of CTL recognizing wildtype peptide, but tumor recognition was not detected.

The binding affinity of peptides for HLA molecules can be determined as described in Example 1, below.

### III.D. Peptide Epitope Binding Motifs and Supermotifs

Through the study of single amino acid substituted antigen analogs and the sequencing of endogenously bound, naturally processed peptides, critical residues required for allele-specific binding to HLA molecules have been identified. The presence of these residues correlates with binding affinity for HLA molecules. The identification of motifs and/or supermotifs that correlate with high and intermediate affinity binding is an important issue with respect to the identification of immunogenic peptide epitopes for the inclusion in a vaccine. Kast *et al. (J. Immunol.* 152:3904-3912, 1994) have shown that motif-bearing peptides account for 90% of the epitopes that bind to allele-specific HLA class I molecules. In this study all possible peptides of 9 amino acids in length and overlapping by eight amino acids (240 peptides), which

cover the entire sequence of the E6 and E7 proteins of human papillomavirus type 16, were evaluated for binding to five allele-specific HLA molecules that are expressed at high frequency among different ethnic groups. This unbiased set of peptides allowed an evaluation of the predictive value of HLA class I motifs. From the set of 240 peptides, 22 peptides were identified that bound to an allele-specific HLA molecule with high or intermediate affinity. Of these 22 peptides, 20 (*i.e.* 91%) were motif-bearing. Thus, this study demonstrates the value of motifs for the identification of peptide epitopes for inclusion in a vaccine: application of motif-based identification techniques will identify about 90% of the potential epitopes in a target antigen protein sequence.

Such peptide epitopes are identified in the Tables described below.

Peptides of the present invention may also comprise epitopes that bind to MHC class II DR molecules. A greater degree of heterogeneity in both size and binding frame position of the motif, relative to the N and C termini of the peptide, exists for class II peptide ligands. This increased heterogeneity of HLA class II peptide ligands is due to the structure of the binding groove of the HLA class II molecule which, unlike its class I counterpart, is open at both ends. Crystallographic analysis of HLA class II DRB\*0101-peptide complexes showed that the major energy of binding is contributed by peptide residues complexed with complementary pockets on the DRB\*0101 molecules. An important anchor residue engages the deepest hydrophobic pocket (*see, e.g.*, Madden, D.R. *Ann. Rev. Immunol.* 13:587, 1995) and is referred to as position 1 (P1). P1 may represent the N-terminal residue of a class II binding peptide epitope, but more typically is flanked towards the N-terminus by one or more residues. Other studies have also pointed to an important role for the peptide residue in the 6<sup>th</sup> position towards the C-terminus, relative to P1, for binding to various DR molecules.

In the past few years evidence has accumulated to demonstrate that a large fraction of HLA class I and class II molecules can be classified into a relatively few supertypes, each characterized by largely overlapping peptide binding repertoires, and consensus structures of the main peptide binding pockets. Thus, peptides of the present invention are identified by any one of several HLA-specific amino acid motifs (*see, e.g.*, Tables I-III), or if the presence of the motif corresponds to the ability to bind several allele-specific HLA antigens, a supermotif. The HLA molecules that bind to peptides that possess a particular amino acid supermotif are collectively referred to as an HLA "supertype."

The peptide motifs and supermotifs described below, and summarized in Tables I-III, provide guidance for the identification and use of peptide epitopes in accordance with the invention.

Examples of peptide epitopes bearing a respective supermotif or motif are included in Tables as designated in the description of each motif or supermotif below. The Tables include a binding affinity ratio listing for some of the peptide epitopes. The ratio may be converted to IC<sub>50</sub> by using the following formula: IC<sub>50</sub> of the standard peptide/ratio = IC<sub>50</sub> of the test peptide (*i.e.*, the peptide epitope). The IC<sub>50</sub> values of standard peptides used to determine binding affinities for Class I peptides are shown in Table IV. The IC<sub>50</sub> values of standard peptides used to determine binding affinities for Class II peptides are shown in Table V. For example, where an HLA-A2.1 motif-bearing peptide shows a relative binding ratio of 0.01 for HLA-A\*0201, the IC<sub>50</sub> value is 500 nM, and where an HLA-A2.1 motif-bearing peptide shows a relative binding ratio of 0.1 for HLA-A\*0201, the IC<sub>50</sub> value is 50 nM.

The peptides used as standards for the binding assays described herein are examples of standards; alternative standard peptides can also be used when performing binding studies.

To obtain the peptide epitope sequences listed in Tables VII-XX, protein sequence data for HPV types 6a, 6b, 11a, 16, 18, 31, 33, 45, and 56 were evaluated for the presence of the designated supermotif or motif. Seven HPV structural and regulatory proteins, E1, E2, E5, E6, E7, L1 and L2 were included in the analysis. E4 was also included in the evaluation of some of the strains. Peptide epitopes can additionally be evaluated on the basis of their conservancy (*i.e.*, the amount of variance) among the available protein sequences for each HPV antigen.

In the Tables, motif- and/or supermotif-bearing amino acids sequences identified in the indicated HPV strains are designated by position number and length of the epitope with reference to the HPV sequences and numbering provided below. For each sequence, the four columns provide the following information: column 1 indicates the HPV strain; column 2 indicates the HPV protein in which the motif-bearing sequence is found, *e.g.*, E1, E2, E4, E5, E6, E7, L1, or L2; column 3 indicates the length of the epitope, or in the case of HLA Class II epitopes, the length of the core sequence; and column 4 designates the amino acid position in the HPV protein sequence that corresponds to the first amino acid residue of the epitope. For those sections of the Tables that include only three columns, corresponding to columns 2, 3, and 4, the HPV strain is indicated in the heading at the top of the page. For example, the first peptide epitope listed in Table VII, *i.e.*, the HLA-A1 supermotif, for HPV 16, protein E1 is a sequence of 10 residues in length starting at position 206. Accordingly, the amino acid sequence of the epitope is AMLAKFKELY.

For HPV strain 11, the number and position listed for protein E5 refers to either the HPV11 E5a or HPV11 E5b sequence set out below. Because the epitope must include the designated motif or supermotif, *e.g.*, HLA-A2, it can readily be determined whether the sequence refers to HPV11 E5a or E5b by checking the amino acid sequences of both E5a and E5b and selecting the sequence that conforms to the motif listed in Table I.

#### HPV STRAINS AND AMINO ACID SEQUENCES OF HPV PROTEINS

##### HPV6A E1

1	MADDSGTENEGSGCTGWMVEAIVQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS	60
30	LEAQALFNRQEQADTHYATVQDLKRKYLGSPPYSPINTIAEAVESEISPRLDIAIKLTRQPK	120
	KVKRRLFQTRELTSYGYGYSEVEAGTGTQVEKHGVPENGGDQEKDTRDIEGEEHTEAE	180
	APTNSVREHAGTAGILELLKCKDLRAALLGKFKECFGLSFIDLIRPFKSDKTTCADWVVA	240
	GFGIHHSISEAFQKLIIEPLSLYAHIQWLTNAWGMVLLVLRPFKNKSRSTVARTLATLLN	300
	IPDNQMLIEPPKIQSGVAALYWFRTGISNASTVIGEAPWITRQTVEHGLADSQFKLTE	360
35	MVQWAYDNDICEESEIAFEYAQRGDFDSNARAFLNSNMQAKYVKDCATMCRHYKHAEMRK	420
	MSIKQWIKHRGSKIIEGTGNWKPIVQFLRHQNIIEFIPFLSKFKLWLHGTPKKNCIAIVGPP	480
	DTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDKVALLDATQPCWIYMDTYMRNL	540
	LDGNPMSIDRKHKALTLIKCPPLLVTNSNIDITKEEKYKYLHTRVTTFTFPNPPFPDRNGN	600
	AVYELSNANWKCFFERLSSSLDIQDSEDEEDGSNSQAFCVPGTVVRTL	649



HPV6A E2

1 MEAIAKRLDACQEQLLELYEENSTDNLNKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP 60  
 PLKVSEAKGHNAIEMQMHLSELLKTEYSMEPWTLQETSYEMWQTPPKRCFKKRGKTVEVK 120  
 FDGCANNTMDYVWVTDVYVQDTSWVKVHSMVDAKGIYYTCGQFKTYVNFVKEAEKYGS 180  
 5 TKQWEVCYGSTVICSPASVSSTTQEVSIPSTTYTPAQSTPVSSTQEDAVQTPPRKRA 240  
 RGVQQSPCNALCVAHIGPVDSGNHNLTNMHDQHQRNNSNSSATPIVQFQGESNCLKCF 300  
 RYRLNDKHRHFLDLISSTWHWASPKAPHKHAIVTVTYHSEEQRQQFLNVVKIPPTIRHXL 360  
 GFMSLHLL 368

10 HPV6A E4

1 MAAQLYVLLHLHLALHKKYPFLNLLHTPPHRPPPLCPQAPRKTQCKRRENEHEESNSHL 60  
 ATPCVWPTLDPWTVETTTSSLTITTSTKEGTTVTVQLRL 99

HPV6A E5

15 1 MEVVPVQIAAGTTSTLILPVIIAFVVCFSIILIVWISDFIVYTSVLVLTLLLYLLLWLL 60  
 LTTPLQFFLLTLLVCYCPALYIHXYIVNTQQ 91

HPV6A E6

1 MESANASTSATTIDQLCKTFNLSMHTLQINCVFCKNALTTAEIYSYAYKQLKVLFRGGYP 60  
 20 YAACACCLEFHHGKINQYRHFDYAGYATTVEEETKQDILDVLIRCYLCHKPLCEVEKVKHI 120  
 LTKARFIKLNCTWKGRCLHCWTTTCMEDMLP 150

HPV6A E7

1 MHGRHVTLKDIVLDLQPPDPVGLHCYEQLVDSSEDEVDEVDGQDSQPLKQHFQIVTCCCG 60  
 25 CDSNVRLVVQCTETDIREVQQLLLGTLDIVCPICAPKT 98

HPV6A L1

1 MWRPSDSTVYVPPNPVSKVVATDAYVTRTNIFYHASSRLLAVGHPYFSIKRANKTVVP 60  
 KVSGYQYRVFKVVLDPNKFALPDSSLFDPITTQRLVWACTGLEVGRGQPLGVGVSGHPFL 120  
 30 NKYDDVENS SGGNPGQDNRVNVGM DYKQTQLCMVGCAPPLGEHWGKGKQCTNTPVQAGD 180  
 CPPLELITSVIQDGMVDTGFGAMNFADLQTNKSDVPIDICGTTCKYPDYLMADPYGD 240  
 RLFFFLLRKEQMFAHFNRAGEVGEVPVDTLIIKSGSNRTSVGSSIYVNTPSGSLVSSEA 300  
 QLFNKPYWLQKAQGHNNGICWGNQLFVTVDTRSTNMTLCASVTTSSTYTNSDYKEYMR 360  
 HVEEYDLQFIFQLCSITLSAEVMAYIHTMNPSVLEDWNFGLSPPNGTLEDYRYVQSQA 420  
 35 ITCQKPTPEKEKPDYKNSLFWEVNLKEKFSSELDQYPLGRKFLQSGYRGRSSIRTGVK 480  
 RPAVSKASAAPKRKRAKTKR 500

HPV6A L2

1 MAHSRARRRRKRASATQLYQTCKLTGTCPPDVIPKVEHNTIADQILKWGSLGVFFGGLGIG 60  
 40 TGSGTGGRGTGYVPLGTSAPKSITSGPMARPPVVVEPVAPSDPSIVSLIEESAIINAGAPE 120  
 IVPPAHGGFTITSSETTTTTPAILDVSVTSHTTTTSIFRNPVFTEPSVTQPQPPVEANGHILI 180

SAPTITSHPIEEIPLDTFVISSSDSGPTSSTPVPGTAPRPRVGLYSRALHQVQVTDPAFL 240  
 STPQRLITYDNPVYEGEDVSVQFSDHSIHNPDEAFMDIIRLHRPAIASRRGLVRYSRIG 300  
 QRGSMHTRSGKHIGARIHYFYDISPIAQAAEEIEMHPLVAAQDDTFDIYAESFEPDINPT 360  
 QHPVTNISDTYLTSTPNTVTQPWGNTTVPLSSIPNDLFLQSGPDITFPTAPMGTPFPSPVT 420  
 5 ALPTGPFVITGSGFYLHPAWYFARKRRKRIPLFFSDVAA 459

HPV6B E1

1 MADDSGTENEGSGCTGWFMEAI VQHPTGTQISDDEDEEVEDSGYDMVDFIDDSNITHNS 60  
 LEAQALFNRQEQADTHYATVQDLKRKYLGSPIVSPINTIAEAVESEISPRLDAIKLTRQPK 120  
 10 KVKRRLFPQTRBLTDSGYGYSEVEAGTGTQVEKHGVPENGGDQEKDTRDIEGEEHTEAE 180  
 APTNSVREHAGTAGILELLKCKDLRAALLGKFKECGLSFIDLIRPFKSDKTTCLDWVVA 240  
 GFGIHHSISEAFQKLIIEPLSLYAHIQWLTNAWGMVLLVLLRFKVNKSRSTVARTLATLLN 300  
 IPENQMLIEPPKIQSGVAALYWFRGTGISNASTVIGEAPWITRQTVIEHGLADSQFKLTE 360  
 MVQWAYDNDICEESEIAFEYAQRGDFDSNARAFNSNMQAKYVKDCATMCRHYKHAEMRK 420  
 15 MSIKQWIKHRGSKIETGNWKPIVQFLRHQNIIEFIPFLTKFKLWLHGTPKKNCIAIVGPP 480  
 DTGKSYFCMSLISFLGGTVISHVNSSSHFWLQPLVDKVALDDATQPCWIYMDTYMRNL 540  
 LDGNPMSIDRKHKALTLIKCPPLLVTSNIDITKEDKYKYLHTRVTTFTFPNPFPPDRNGN 600  
 AVYELSNNTWKCFERLSSSLDIQDSEDEEDGSNSQAFRCVPGTVVRTL 649

20 HPV6B E2

1 MEAIAKRLDACQEQLLELYEENSTDLHKHVLHWKCMRHESVLLYKAKQMGLSHIGMQVVP 60  
 PLKVSEAKGHNAIEMQMHLLESLRTEYSMEPWTLOETS YEMWQTPPKRCFKKRGKTVEVK 120  
 FDGCANNTMDYVWTDVYVQDNDTWVKVHSMVDAKGIYYTCGQFKTYVNFVKEAEKYGS 180  
 TKHWEVCYGSTVICSPASVSSTTQEVSIPESTTYTPAQTSTLVSSSTKEDAVQTPPRKRA 240  
 25 RGVQQSPCNALCVAHIGPVDSGNHNLTNMHDQHQRNNSNSSATPIVQFQGESNCLKCF 300  
 RYRLNDRHRHLFDLISSTWHWASSKAPHKHAIVTVTYDSEQRQQFLDVVKIPPTISHKL 360  
 GFMSLHLL 368

HPV6B E4

30 1 MGAPNIGKYVMAAQLYVLLHLYLALHKKYPFLNLLHTPPHRPPPLCPQAPRKTQCKRRLG 60  
 NEHEESNSPLATPCVWPTLDPWTVETTTSSLTITTSTKDGTTVTVQLRL 109

HPV6B E5A

1 MEVVPVQIAAGTTSTFILPVIIAFVVCVFSIILIVWISEFIVYTSVLVLTLLLYLLLWLL 60  
 35 LTTPLOQFFLLTLLVCYCPALYIHYIIVTTQQ 91

HPV6B E5B

1 MMLTCQFNDGDTWLGLWLLCAFI VGMGLLLLHYRAVQGDKHTKCKKCNKHN CNDDYVTM 60  
 HYTTDGDYIYMN 72

40

HPV6B E6

1 MESANASTSATTIDQLCKTFNLSMHTLQINCVFCKNALTTAEIYSYAYKHLKVLFRGGYP 60  
 YAACACCLEPHGKINQYRHFYAGYATTVEEETKQDILDVLRICYLCHKPLCEVEKVKHI 120  
 LTKARFIKLNCTWKGRCLHCWTTTCMEDMLP 150

5

HPV6B E7

1 MHGRHVTLKDIVLDLQPPDPVGLHCYEQLVDSSSEDEVDEVDGQDSQPLKQHFQIVTCCCG 60  
 CDSNVRLVVQCTETDIREVQQLLLGTLNIVCPICAPKT 98

10 HPV6A L1

1 MWRPSDSTVYVPPNPVSKVVATDAYVTRTNIFYHASSRLLAVGHPYFSIKRANKTVVP 60  
 KVSGYQYRVFKVVLDPNKFALPDSSLFDPTTQRLVWACTGLEVGRGQPLGVGVSGHPFL 120  
 NKYDDVENSNGSGGNPGQDNRVNVGMDYKQTQLCMVGCAPPLGEHWGKGKQCTNTPVQAGD 180  
 CPPLLELITSVIQDGMVDTGFGAMNFADLQTNKSDVPIDICGTTCKYPDYLQMAADPYGD 240  
 RLFFFLRKEQMFAHFHFNRAAGEVGEVDPDTLIIKSGNRTSVGSSIIYVNTPSGSLVSSEA 300  
 QLENKPYWLQKAQGHNNIGICWGNQLFVTVDTTSTNMTLCASVTTSSSTYNSDYKEYMR 360  
 HVEEYDLQFIFQLCSITLSAEVMAYIHTMNPSVLEDWNFGLSPPPNGTLEDYRYVQSQA 420  
 ITCQKPTPEKEKPDYPKNLSFWEVNLKEKFSSELDQYPLGRKFLQSGYRGRSSIRTGVK 480  
 RPAVSKASAAPKRKRKRAKTKR 500

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HPV6B L2

1 MAHSRARRRRKRASATQLYQTCKLTGTCPDPVIPKVEHNTIADQILKWGSLGVFFGGLGIG 60  
 TGSSTGGRTGYVPLQTSAPKPSITSGPMARPPVVVEPVAPSDPSIVSLIEESAIINAGAPE 120  
 IVPPAHGGFTTISSETTTTTPAILDVSVTSHTTTISFRNPVFTEPSVTQPPVEANGHILI 180  
 SAPTVTSHPIEIEIPLDTFVVSSSDSGPTSSTPVPGTAPRPRVGLYSRALHQVQVTDPAFL 240  
 STPQRLITYDNPVYEGEDVSVQFSDHSIHNAPEAFMDIIRLHRPAIASRRGLVRYSRIG 300  
 QRGSMHTRSGKHIGARIHYFYDISPIAQAAEEIEMHPLVAAQDDTFDIYAESFEPGINPT 360  
 QHPVTNISDTYLTSTPNTVTQPGWNTTVPLSLPNDLFLQSGPDITFPTAPMGTPFSPVTP 420  
 ALPTGPVPFITGSGFYLHPAWYFARKRRKRIPLFFSDVAA 453

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HPV11 E1

1 MADDSGTENEGSGCTGWMVEAIVEHTTGTQISEDEEEVEDESGYDMVDFIDDRHITQNS 60  
 VEAQALFNRQEADAHYATVQDLKRKYLGSPIVSPISNVANAVESEISPRLDAIKLTTQPK 120  
 KVKRRLFETRELTDSGYGYSEVEAATQVEKHGDPENGGDGQERDTGRDIEGEGVEHREAE 180  
 AVDDSTREHADTSGILELLKCKDIRSTLHGKFKDCFGLSFVDLIRPFKSDRTTCADWVVA 240  
 GFGIHHSIADAFQKLIPLSLYAHIQWLTNANGMVLVLLVIRFKVNSRCTVARTLGTLLN 300  
 IPENHMLIEPPKIQSGVRALYWFRGTGISNASTVIGEAPEWITRQTVIEHSLADSQFKLTE 360  
 MVQWAYDNDICEESEIAFEYAQRGDFDSNARAFNLSNMQAKYVKDCAIMCRHYKHAEMKK 420  
 MSIKQWIKYRGTKVDSVGNWKPVQFLRHQNIIEFIPFLSKLKLWLHGTTPKKNCIAIVGPP 480  
 DTGKSCFCMSLIKFLGGTVISYVNSCSHFWLQPLTDAKVALLDDATQPCWTYMDTYMRNL 540  
 LDGNPMSIDRKHRALTLIKCPPLLVTSNIDISKEEKYKYLHSRVTTFTFPNPPFPDRNGN 600

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AVYELSDANWKCFERLSSSLDIEDSEDEEDGSNSQAFRCVPGSVVRTL 649

HPV11 E2

1 MEAIAKRLDACQDQLLELYEENSIDIHKHIMHWKCIRLESVLLHKAKQMGLSHIGLQVVP 60  
 5 PLTVSETKGHNAIEMQMHLLESIAKTQYGVPEWTLQDTSYEMWLTPPKRCFKKQGNTVEVK 120  
 FDGCEDNVMEYVVWTHIYLQDNDSWVKVTSSVDAKGIYYTCGQFKTYVNFNKEAQKYGS 180  
 TNHWEVCYGSTVICSPASVSSTVREVSIAEPTTYTPAQTTAPTVSACTTEDGVSAPPRKR 240  
 ARGPSTNNTLCVANIRSVSTINNIVTDNYNKHQRRNNCHSAATPIVQLQGDSNCLKCFR 300  
 YRLNDKYKHLFELASSTWHWASPEAPHKNAIVTLTYSSEEQRQQFLNSVKIPPTIRHKVG 360  
 10 FMSLHLL 367

HPV11 E4

1 MUVPIIGKYVMAAQLYVLLHLYLALYEKYPLLNLHTPPHRPPPLQCPPAPRKTACRRRL 60  
 GSEHVDRLPTTPCVWPTSDPWTQSTTSSLTITTSTKEGTTVTVQLRL 108

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HPV11 E5A

1 MEVVVPQIAAATTTTLILPVVIAFAVCILSIVLIILISDFVVYTSVLVLTLLLYLLWLL 60  
 LTTPLQFFLLTLCVCYFPAFYIHIYIVQTQQ 91

20 HPV11 E5B

1 MVMLTCHLNDGDTWLFLWLFTAFVAVLGLLLLHYRAVHGTEKTKCAKCKSNRNTTVDYV 60  
 YMSHGDNGDYVYMN 74

HPV11 E6

25 1 MESKDASTSATSIDQLCKTFNLSLHTLQIQCVFCRNALTTAEIYAYAYKNLKVVRDNFP 60  
 FAACACCLELOGKINQYRHFNYAAYAPTVEETNEDILKVLIRCYLCHKPLCEIEKLKHI 120  
 LGKARFIKLNNQWKGRCCLHCWTTCCMEDLLP 150

HPV11 E7

30 1 MHGRLVTLKDIDLQPPDPVGLHCYEQLEDSSSEDEVDKVDKQDAQPLTQHYQILTCCCG 60  
 CDSNVRLVVECTDGDIRQLQDLLLGTNLNIVCPICAPKP 98

HPV11 L1

1 MWRPSDSTVYVPPNPVSKVVATDAYVKRTNIFYHASSRLLAVGHPYYSIKKVNTTVVP 60  
 35 KVSGYQYRVFKVVLDPDNKFALPDSSLFDPPTQRLVWACTGLEVGRGQPLGVGVSGHPLL 120  
 NKYDDVENSNGGYGGNPGQDNRVNVGMDYKQTQLCMVGCAPPLGEHWGKGTQCSNTSVQNG 180  
 DCPPLELITSVIQDGMVDTGFGAMNFADLQTNKSDVPLDICGTVCYKYPDYLMQAADPYG 240  
 DRLFFYLKKEQMFARHFFNRAGTVGEPVPDDLLVKGGNNRSSVASSIYVHTPSGSLVSSE 300  
 AQLFNKPYWLQKAQGHNNGICWGNHFLVTVVDTTTRSTNMTLCASVSKSATYTNSDYKEYM 360  
 40 RHVEEFDLQFIFQLCSITLSAEVMAYIHTMNPVLEDWNFGLSPPPNGTLEDTRYVQSQ 420  
 AITCQKPTPEKEKQDPYKDMSPWEVNLKEKFSSELDQFPLGRKFLLQSGYRGRTSARTGI 480

KRPAVSKPSTAPKRKRTKTKK 501

HPV11 L2

1 MKPRARRRRKRASATQLYQTCKATGTCTPPDVIPKVEHTTIADQILKWGSLGVFFGGLGIGT 60  
 5 GAGSGGRAGYIPLGSSPKPAITGGPAARPPVLVEPVAPSDPSIVSLIEESAIINAGAPEV 120  
 VPPTQGGFTITSSSESTTPAILDVSVTNHTTTTSVFQNPLPTEPSVIQPPVEASGHILIS 180  
 APTITSQHVEDIPLDTFVVSSSDSGPTSSTPLPRAFP RPRVGLYSRALQQVQVTDPAFLS 240  
 TPQRLVTYDNPVYEGEDVSLQFTHESIHNAPDEAFMDIIRLHRPAITSRRGLVRF SRIGQ 300  
 RGSMTYTRSGQHIGARIHYFQDISPVTQAAEEIELHPLVAAENDTFDIYAEPFDPIPDVQ 360  
 10 HSVTQSYLTSTPNTLSQSWGNTTVPLSIPSDWFVQSGPDITFPTASMGTPFPSPVTPALPT 420  
 GPVFITGSDFYLHPTWYFARRRRKRIPLFPTDVAA 455

HPV16 E1

1 MADPAGTNGEETGTCNGWFYVEAVVEKKTGDAISDDENENDSDTGEDLVDFIVNDNDYLT 60  
 15 QAETETAHALFTAQEAQHRDAVQVLKRKYLVSPLSDISGCVDNNISPRLKAICIEKQSR 120  
 AAKRRLFESEDSGYGNTVEVETQQMLQVEGRHETETPCSQYSGSGGGCSQYSSGSGGEGV 180  
 SERHTICQTPLTNILNVLKTSNAKAAMLAKFKELYGVSFSELVRPFKSNKSTCCDW CIAA 240  
 FGLTPSIADSIKTLQYCLYLHIQSLACSWGMVVLVRYKCGKNRETIEKLLSKLLCV 300  
 SPMCMMEIPPKLRSTAAALYWKYTGISNISEVYGDTPEWIQRQTVLQHSFNDCTFELSQM 360  
 20 VQWAYDNDIVDDSEIAYKYAQLADTNSNASAFKLSNSQAKIVKDCATMCRHYKRAEKKQM 420  
 SMSQWIKYRCRDVDDGGDWKQIVMFLRYQGVFMSFLTALKRFLQGIPKKNCILLYGAAN 480  
 TGKSLFGMSLMKFLQGSVICFVNSKSHFWLQPLADAKIGMLDDATVPCWNYIDDNL RNAL 540  
 DGNLVSMDDVKHRPLVQLKCPPLLITSNINAGTDSRWPYLNRLVVFTFPNEFPFDENG NP 600  
 VYELNDKNWKSFFSRTWSRLSLHEDEDKENDGDSLPTFKCVSGQNTNTL 649

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HPV16 E2 Accession number W2WLHS

1 METLCQRLNVCQDKILTHYENDSTDLRDHIDYWKHMRLECAIYYKAREMGFKHINHQVVP 60  
 TLAVSKNKALQAIELQLTLETIYNSQYSNEKWTLDVSLLEVYLTAPTGCICKHGYTVEVQ 120  
 FDGDICNTMHYTNWTHIYICEEASVTVEGQVDYVGLYYVHEGIRTYFVQFKDDAEKYSK 180  
 30 NKVWEVHAGGQVILCPTSVFSSNEVSSPEIIROHLANHPAATHTKAVALGTEETQTTIQR 240  
 PRSEPDTGNPCHTTKLLHRDSVDSAPILTAFNSSHKGRINCNSNTTPIVHLKGDANTLKC 300  
 LRYRPFKKHCTLYTAVSSTWHWTGHNVKHKSIAVTLTYDSEWQRDQFLSQVKIPKTI TVST 360  
 GFMSI 365

35 HPV16 E5 Accession number W5WLHS

1 MTNLDTASTTLLACFLLCFVLLCVCLLIRPLLSVSTYTSLLIILVLLWITAASAFRCF 60  
 IVYIIFVYIPLFLIHTHARFLIT 83

HPV16 E6

40 1 MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFAFRDL CIV 60  
 YRDGNPYAVCDKCLKFYISKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPE 120

EKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL 158

HPV16 E7

1 MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEBEDEIDGPAGQAEPRAHYNIVTFCKK 60  
5 CDSTLRCLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP 98

HPV16 L1 Accession number AAD33259

1 MQVTFIYILVITCYENDVNVIHFFQMSLWLPSEATVYLPPVPVSKVVSTDEYVARTNIY 60  
YHAGTSRLLAVGHPYFPPIKKPNNNKILVPKVSGLQYRVFRIHLDPNKFPGPDTSFYND 120  
10 TQRLVWACVGVGGRGQPLGVGISGHPLLNKLDLTENASAYAANAGVDNRECISMDYKQT 180  
QLCLIGCKPPIGEHWGKSGPCTNVAVNPGDCPPLELINTVIQDGMVDTGFGAMDFTTLQ 240  
ANKSEVPLDICTSICKYPDYIKMVSEPYGDSLFFYLRRREQMFVRHLFNRAGAVGENVPDD 300  
LYIKGSGSTANLASSNYFPTPSGSMVTSDAQIFNKPYWLQRAQGHNNGICWGNQLFVTVV 360  
DTRSTNMSLCAAISTSETTYKNTNFKEYLRHGEEDLQFIFQLCKITLTADVMTYIHS 420  
15 NSTILEDWNFGLQPPPGGTLEDTYRFVTSQAIACQKHTPPAPKEDPLKKYTFWEVNLKEK 480  
FSADLDQFPPLGRKFLQAGLKAKPKFTLGRKATPTTSSTSTAKRKRKL 531

HPV16 L2 Accession number AAD33258

1 MRHKRSARKTRKASATQLYKTCKQAGTCPPDIIPKVEGKTIADQILQYSGMGVFFGGLGI 60  
20 GTGSGTGGRGTGYIPLGTRPPTATDTLAPVRPPLTVDPVGPSPSIVSLVEETSFDAGAP 120  
TSVPSIPPDVSGFSITSTDTTPAILDINNVTVTTHNNPTFTDPSVLQPPTPAETGGH 180  
FTLSSSTISTHNYEIPMDTFIVSTNPNTVTSSTPIPGSRPVARLGLYSRTTQQVKVDP 240  
AFITTPTKLITYDNPAYEGIDVDNTLYFSSNDNSINIAPDPDFLDIVALHRPALTSRRTG 300  
IRYSRIGNKQTLRTRSGKSIGAKVHYYYDFSTIDSAAEIELQTITPSTYTTTSHAALPTS 360  
25 INNGLYDIYADDFITDTSTTPVPSVPSTSLSGYIPANTTIPFGGAYNIPLVSGPDIPINI 420  
TDQAPSLIPIVPGSPQYTIADAGDFYLHPSYYMLRKRKRRLPYFFSDVSLAA 473

HPV18 E1

1 MADPEGTDGEGTGCGWIFYVQAIVDKKTGDVISDDDEDENATDTGSDMVDFIDTQGTFCQ 60  
30 AELETAQALFHAQEVHNDQVLHVLKRKFAGGSTENSPLGERLEVDTLSRLQEISLNS 120  
GQKKAKRRLFTISDSGYGCSEVEATQIQVTTNGEHGGNVCSGGSTEADNNGGTEGNNSSV 180  
DGTSDNSNIENVNPQCTIAQLKDLLKVNKQGAMLAVFKDTYGLSFTDLVRNFKSDKTC 240  
TDWVTAIFGVNPTIAEGFKTLIQPFILYAHIQCLDCKWGLILALLRYKCGKSRLTVAKG 300  
LSTLLHVPETCMLIQPPKLRSVAALYWYRTGISNISEVMGDTPEWIRLTIIQHIGIDDS 360  
35 NFDLSEMVOQAFDNELTDESMAFEYALLADSNSNAAFLKSNCQAKYLKDCATMCKHYR 420  
RAQKRQMNMSQWIRFRCSKIDEGGDWRPIVQFLRYQQIEFITFLGALKSFLKGTTPKNCL 480  
VFCGPANTGKSYFGMSFIHQGAVISFVNSTSHFWLEPLTDTKVAMLDATTTCWTFYD 540  
TYMRNALDGNPISIDRKHKPLIQLKCPPILLTTNIHPAKDNRWPYLESRITVFEFPNAPP 600  
FDKNGNPVYEINDKNWKCFFERTWSRLDLHEEEEDADTEGNPFGTFKLRAQONHRPL 657

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HPV18 E2 Accession number W2WL18

1 MQTPKETLSERLSCVQDKIIDHYENDSKDIDSQIQYWQLIRWENAIFFAAREHGIQTLNH 60  
 QVVPAYNISKSKAHKAIELOMALQGLAQSRKTEDWTLQDTCEELWNTTEPTHCFKKGQT 120  
 VQVYFDGNKONCMTYVAWDSVYYMTDAGTWDKTATCVSHRGLYYVKEGYNTFYIEFKSEC 180  
 5 EKYGNTGTWEVHFGNNVIDCNDSDMCSTSDDTVSAQQLVKQLQHTPSPYSSVTVSVGTAKTY 240  
 GQTSAAATRPGHCGLAQKQHCQFVNPLGAATPTGNNKRRKLCSENTTPIIHLKGDNRSLK 300  
 CLRYRLRKHSDDHYRDISSTWHWTGAGNEKTGILTVTYHSETQRTKFLNTVAIPDSVQILV 360  
 GYMTM 365

10 HPV18 E5 Accession number W5WL18

1 MSLSLIFLFCFCVCMYVCHVPLLPVCMCAWVLFVYIVVITSPATAFTVYVFCFLLP 60  
 MLLLHIHAILSLQ 73

HPV18 E6

15 1 MARFEDPTRRYPKLPDLCTELNTSLQDIBITCVYCKTVLELTVFEFAFKDLFVVYRDSI 60  
 PHAACHKCIDFYSRIRELRHYSVYGDITLTKLNTGLYNLLIRCLRCQKPLNPAEKLRLH 120  
 LNEKRRFHNIAAGHYRGQCHSCCNRRARQERLQRRRETQV 158

HPV18 E7

20 1 MHGPKATLQDIVLHLEPQNEIPVDLLCHEQLSDSEEENDEIDGVNHQHLPARRAEPQRHT 60  
 MLCMCKCEARIKLVVESSADDLRAFQQLFLNTLSFVCPWCASQQ 105

HPV18 L1 Accession number CAA28671

1 MCLYTRVLILHYHLLPLYGPLYHPRPLPLHSILVYMHIIICGHYIILFLRNVNVFPIFL 60  
 25 QMALWRPSDNTVYLPSPSVARVNTDDYVTPTSIFYHAGSSRLTLVGNPYFRVPAGGGNK 120  
 QDIPKVSAYQYRVFRVQLPDPNKFGLPDTSIYNPETQRLVWACAGVEIGRGQPLGVGLSG 180  
 HPFYNKLDDESSHAATSNVSEDVRDNVSDYKQTLCLGCAPAGEHWAKGTACKSRP 240  
 LSQGDCEPPELKNVTLEDGDMVDTGYGAMDFSTLQDTKCEVPLDICQSICKYPDYLOMSA 300  
 DPYGDMSFFCLRREQLFARHFWRNAGTMGDTVPQSLYIKGTGMPASPGSCVYSPSPSGSI 360  
 30 VTSDSQLFNKPYWLHKAQGHNNQVCWHNQLFVTVDTPSTNLITCASTQSPVPGQYDAT 420  
 KFKQYSRHEEYDLQFIFQLCTITLTADVMSYIHSMNSSILEDWNFGVPPPTTSLVDY 480  
 RFVQSVAITCQKDAAPAKNDPYDKLFKFNVDLKEKFSLDLDQYPLGRKFLVQAGLRRKP 540  
 TIGPRKRSAPSATTSSKPAKRVRVRAR 568

35 HPV18 L2 Accession number P2WL18

1 MVSHRAARRKRASVTDLYKTCQSGTCPPDVVPKVEGTTLADKILQWSSLGIFLGLGIG 60  
 TGSGTGGRGTGYIPLGGRSNTVVDVGPTRPFPVIEPVGPTDPSIVTLIEDSSVVTSGAPRP 120  
 TFTGTSGFDITSAGTTTTPAVLDITPSSTSVSISTTNFTNPAFSDPSIIEVPQTGEVAGNV 180  
 FVGTPSTSGTHGYEEIPLQTFASSGTGEEPISSTPLPTVRRVAGPRLYSRAYQQVSVANPE 240  
 40 FLTRPSSLITYDNPAFEPVDTTLTDFDPRSDVPSDFMDIIRLHRPALTSRRGTVRFSRLG 300  
 QRATMFRSGTQIGARVHFYHDISPIAPSFEYIELQPLVSATEDNDLFDIYADDMDPAVP 360

VPSRSTTSFAFFKYSPTISSASSYSNVTVPLTSSWDVPVYTGPDITLPSTTSVWPIVSPT 420  
 APASTQYIGIHGTHYYLWPLYFYIPKKRKRVPYFFADGFVAA 462

HPV31 E1 Accession number W1WL31

5 1 MADPAGTDGEGTGCNGWFYVEAVIDRQTGDNISEDENEDSSDTGEDMVD FIDNCNVYNNQ 60  
 AEAETAQALFHAQAEAEHAEAVQVLKRKYVGSPLSDISSCVDYNISPRKKAICIENNSKT 120  
 AKRRLFELPDSDGYGNTETVETQQMVQVEEQQTTLSCNGSDGTHSERENETPTRNILQVLKT 180  
 SNGKAAMLGKFKELYGVSMELIRPFQSNKSTCTDWCVAAGVTGTVAEGFKTLLQPYCL 240  
 YCHLQSLACSWGVMMLVRFKCAKNRITIEKLLKLCISTNCMLIQPPKLRSTAAALY 300  
 10 WYRTGMSNISDVYGETPEWIERQTVLQHSFNDDTTFDLSQMVQWAYDNDVMDSEIAYKYA 360  
 QLADSDSNACAFKLSNSQAKIVKDCGTMCRHYKRAEKQMSMGQWIKSRCDKVSDEGDWR 420  
 DIVKFLRYQQIEFVSFLSALKLFLKGVPKKNCILIHGAPNTGKSYFGMSLISFLQGCIIIS 480  
 YANSKSHFWLQPLADAKIGMLDDATTPCWHYIDNYLRNALDGNPVSIDVKHKALMQLKCP 540  
 PLLITSNINAGKDDRWPYLHSRLVVFPPNPPFPDKNGNPVYELSDKNWKSFFSRTWCRL 600  
 15 NLHBEEDKENDGDSFSTFKCVSGQNIRTL 629

HPV31 E2 Accession number W2WL31

1 METLSQRLNVCQDKILEHYENDSKRLCDHIDYWKHIRLECVL MYKAREMGHISINHQVVP 60  
 ALSVSKAKALQAIELQMMLETLNTEYKNE DWTMQQTSLELYLTAPTGCLKKHGYTVEVQ 120  
 20 FDGDVHNTMHTNWKFIYLCIDGQCTVVEGQVNCKGIYVHEGHITYFVNFTEEAKKYGT 180  
 GKKWEVHAGGQVIVFPESVFSSEISFAGIVTKLPTANNTTTSNSKTCALGTSEGVRRTAT 240  
 TSTKRPRTEPEHRNTHHPNKLRLGDSVDSVNCGVISAACTNQTRAVSCPATTPIIHLKG 300  
 DANILKCLRYRLSKYKQLYEQVSSWHTCTDGKHKNIAIVTLTYISTSQRDDFLNTVKIP 360  
 NTVSVSTGYMTI 372

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HPV31 E5 Accession number W5WL31

1 MIELNISTVSIVLCFLCFCVLLFVCLVIRPLVLSVSVYATLLLLIVILWVIATSPLRCF 60  
 CIYVVFYIPLFVIHTHASFLSQQ 84

30 HPV31 E6 Accession number W6WL31

1 MFKNPAERPRKLHELSSALEIPYDELRLNCVYCKGQLTETEVLDFAFTDLTIVYRDDTPH 60  
 GVCTKCLRFYSKVSEFRWYRYSVYGTTELEKLTNKGICDLLIRCITCQRPLCPEEKQRHLD 120  
 KKKRFHNIGGRWTGRCIACWRRPRRTETQV 149

35 HPV31 E7 Accession number W7WL31

1 MRGETPTLQDYVLDLQPEATDLHCYEQLPDSSDEEDVIDSPAGQAEPTDSNYNIIVTFCCQ 60  
 CKSTLRCLCVQSTQVDIRILQELLMGSFGIVCPNCSTRL 98

HPV31 L1 Accession number P1WL31

40 1 MSLWRPSEATVYLPPVPVSKVSTDEYVTRTNIYYHAGSARLLTVGHPYYSIPKSDNPCK 60  
 IIVPKVSGLQYRVFRVRLPDPNKFPGPDTSFYNPETQRLVWACVGLVGRGQPLGVGISG 120



HPLLNNKFDDENSNRYAGGPGTDNRECISMDYKQTQLCLLGCKPPIGEHWGKGS PCSNNA 180  
 ITPGDCPPLELKNSVIQDGMVDTGFGAMDFTALQDTKSNVPLDICNSICKYPDYLMVA 240  
 EPYGDTLFFYLRRQMFVRHFFNRSGTVGESVPTDLYIKGSGSTATLANSTYFPTPSGSM 300  
 VTSDAQIFNKPYWMQRAQGHNNGICWGNQLFVTVVDTRSTNMSVCAAIANSDDTFKSSN 360  
 5 FKEYLRHGEEFDLQFIFQLCKITLSADIMTYIHSMPAILEDWNFGLTTPPSGSLED TYR 420  
 FVTSQAITCQKTAPQKPKEDPFKDYVFEVNLKEKFSADLDQFPLGRKFLLQAGYRARP 480  
 FKAGKRSAPSASTTTPAKRKKTKK 504.

HPV31 L2 Accession number P2WL31

10 1 MRSKRSTKRTRKASATQLYQTCKAAGTCPSDVIPKIEHTTIADQILRYGSMGVFFGGLGI 60  
 GSGSGTGGRTGYVPLSTRPSTVSEASIPRPVSIDPVGPLDPSIVSLVEESGIVDVGAP 120  
 APIPHPPPTTSGFDIATTADTTPAILDVTSVSTHENPTFTDPSVLQPPTPAETSGHLLLS 180  
 SSISTHNYEEIPMDTFIVSTNNENITSSTPIPGVRRPARLGLYSKATQQVKVIDPTFLSA 240  
 PKQLITYENPAYETVNAEESLYFSNTSHNIAPDPDFLDIIALHRPALTSRRNTVRYSLRG 300  
 15 NKQTLRTRSGATIGARVHYDDISSINPAGESIEMQPLGASATTTSTLNDGLYDIYADTD 360  
 FTVDTPATHNVSPSTAVQSTSASVAYVPTNTTVPLSTGFDIPIFSGPDVPIEHAPTQVFP 420  
 FPLAPTTQVSIFVDGGDFYLHPSYYMLKRRRKRVSYFFTDVSVAA 466

HPV45 E1 Accession number S36563

20 1 MADPEGTDGEGTGCNGWFFVETIVEKKTGDVISDDDETATDTGSDMVD FIDTQLSICEQ 60  
 AEQETAQALFHAQEVQNDAAQVLHLLKRFAGGSKENSPLGEQLSVDTDLSRLQEISLNS 120  
 GHKKAKRRLFTISDSGYGCSEVEAAETQVTVNTNAENGGSVHSTQSSGGDSSDNAENVDP 180  
 HCSITELKELLQASNKKAAMLAVFKDIYGLSFTDLVRNFKSDKTCTDWVMAIFGVNPTV 240  
 AEGFKTLIKPATLYAHIQCLDCKWGVLLIALLRYKCGKNRLTVAKGLSTLLHVPETCMLI 300  
 25 EPPKLRSSVAALYWYRTGISNISEVSGDTPWEIQRLTIIQHGIIDSNFDLSDMVQWAFDN 360  
 DLTDESDMAFQYAQLADCNSNAAFLKSNCAKYLKDCAVMCRHYKRAQKRQMNMSQWIK 420  
 YRCSKIDEGGDWRPIVQFLRYQGVEFISFLRALKEFLKGT PKKNCILLYGPANTGKSYFG 480  
 MSFIHFLQGAIIISFVNSNSHFLEPLADTKVAMLDDATHTCWTYFDNYMRNALDGNPISI 540  
 DRKHKPLLQLKCPPILLTSNIDPAKDNKWPYLESRVTVFTFPHAFPFDKNGNPVYEINDK 600  
 30 NWKCFERTWSRLDLHEDDEDADTEGIPFGTFKCVTGQNTPL 643

HPV45 E2 Accession number S36564

MKMQTPKESLSERLSALQDKILDHYENDSKDINSQISYWQLIRLENAILFTAREHGITKL 60  
 NHQVVPINISKSKAHKAIELQMAKGLAQSKYNNEEWTLQDTCEELWNTEPSQCFKKGG 120  
 35 KTVHVFYFDGNKDCNMNYVWDSIYYITETGIWDKTAACVSYWGVYIKDGDTTYVQFKS 180  
 ECEKYGNSNTWEVQYGGNVIDCNDMSMCSTSDTVSATQIVRQLQHASTSTPKTASVGTPK 240  
 PHIQTPATKRPRQCGLTEQHHGRVNTHVHNPLLCSSTSNNKRRKVCSGNTTPIIHLKGDK 300  
 NSLKCLRYRLRKYADHYSEISSTWHWTGCNKNTGILTVTYNSEVQRNTFLDVVTIPNSVQ 360  
 ISVGYMTI 368

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HPV45 E6 Accession number CAB44706

1 MARFDDPTQRPYKLPDLCTELNTSLQDVSIACVYCKATLERTEVYQFAFKDLFIVYRDCI 60  
 AYAACHKCIDFYSRIRELRYYSNSVYGETLEKITNTELYNLLIRCLRCQKPLNPAEKRRH 120  
 LKDKRRFHSHIAGQYRGQCNTCCDQARQERLRRRRETQV 158

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HPV45 E7 Accession number CAB44707

1 MHGPRATLQEIVLHLEPQNELDPVLLCYEQLSESEEEENDEADGVSHAQLPARRAEPQRH 60  
 KILCVCKCDGRIELTVESSADDLRTLQQFLSTLSFVCPWCATNQ 106

10 HPV45 L1 Accession number CAB44705

1 MAHNIIYGHGIIIFLKNVNVFPIFLQMALWRPSDSTVYLPPPSVARVVNTDDYVSRTSIF 60  
 YHAGSSRLLTVGPNPYFRVVPSPGAGNKQAVPKVSAYQYRVFRVALPDNKFGLPDSTIYNP 120  
 ETQRLVWACVGMIEGRGQPLGIGLSGHPFYNKLDOTESAHAATAVITQDVRDNVSDYKQ 180  
 TQLCILGCVPAIGEHWAKGTLCCKPAQLQPGDCPPLELKNITIEDGDMVDTGYGAMDFSTL 240  
 15 QDTKCEVPLDICQSICKYPDYQLMSADPYGDSMFFCLRREQLFARHFWNRAGVMGDTVPT 300  
 DLYIKGTSANMRETPGSCVYSPSPSGSITSDSQLFNKPYWLHKAQGHNNGICWHNQLFV 360  
 TVVDTRSTNLTLCASTQNPVPNTYDPTKFKHYSRHVEEYDLQFIFQLCTITLTAEVMSY 420  
 IHSMNSSILENWNFGVPPPTTSLVDTYRFVQSVAVTCQKDTTPPEKQDPYDKLKFWTVD 480  
 LKEKFSSDLQYPLGRKFLVQAGLRRRPTIGPRKRPAASTSTASRPKRVRIRSKK 536

20

HPV45 L2 Accession number S36565

1 MVSHRAARRKRASATDLYRTCKQSGTCPPDVINKVEGTTLADKILQWSSLGIFLGGIG 60  
 TGSGSGGRTGYVPLGGRSNTVVDVGPTRPVVIIEVPGPTDPSIVTLVEDSSVVASGAPVP 120  
 TFTGTSGFEITSSGTTTTPAVLDITPTVDSVSISSSTFTNPAFSDPSIIEVPQTGEVSGNI 180  
 25 FVGTPTS GSHGYEEIPLQTFASSGSGTEPISSTPLPTVRRVRGPRLYSRANQQVRVSTSQ 240  
 FLTHPSSLVTFDNPAYEPLDTLSFEPTSNVPDSDFMDIIRLHRPALSSRRGTVRFSRLG 300  
 QRATMPTRSKGQIGGRVHFYHDISPIAATEEIELQPLISATNDSDLFDVYADFPFPASTT 360  
 PSTIHKSFYTPKYSLTMPSTAASSYSNVTVPLTSAWDVPIYTGPDIIILPSHTPMWPSTSP 420  
 TNASTTTYIGIHGTQYYLWPWYYYFPKKRKRIPIYFFADGFVAA 463

30

HPV33 E1 Accession number W1WL33

1 MADPEGTNGAGMGCTGWFEVEAVIERRTGDNISEDEDEDETADDSGTDLLEFIDDSMENSIO 60  
 ADTEAARALFNIQEGEDDLNAVCAKLRKFAACSQSAEDVVDRAANPCRTSINKNKECTY 120  
 RKRKIDELEDSGYGNTEVETQQMVOQVESQNGDTNLNDLESSGVGDDSEVSCETNVDSCE 180  
 35 NVTLQEISNVLHSSNTKANILYKFEAYGISFMELVRPFKSDKTSCTDWCITGYGISPSV 240  
 AESLKVLIKQHSLYTHLQCLTCDRGIIILLIRFRCSKNRLTVAKLMSNLLSIPETCMVI 300  
 EPPKLRSTCALYWFRTAMSNISDVQGTTPPEWIDRLTVLQHSFNDNIFDLSEMVQWAYDN 360  
 ELTDDSDIAYYYAQLADSNSNAAAFKLSNSQAKIVKDCGIMCRHYKKAERKMSIGQWIIQ 420  
 SRCEKTNDDGNNRPVQLLRYQNIETAFLGAFKKFLKGIPKKSCLICGPANTGKSYFG 480  
 40 MSLIQFLKGCVISCVNSKSHFWLQPLSDAKIGMIDDVTPISWTYIDDYMRNALDGNEISI 540  
 DVKHALVQLKCPPLLLTSNTNAGTDSRWPYLHSRLTVFEFKNPFPDENGPNVYAINDE 600

NWKSFFSRTWCKLDLIEEDKENHGGNISTFKCSAGENTRSLRS 644

HPV33 E2 Accession number W2WL33

1 MEEISARLNAVQEKILDLYEADKTDLPSQIEHWKLIRMECALLYTAKQMGFSHLCHQVVP 60  
 5 SLLASKTKAFQVIELQMALETLSKSQYSTSQWTLQQTSLLEVWLCEPPKCFKKQGETVTVQ 120  
 YDNDKKNMTDYNWGEIYIIIEEDTCTMVTGKVDYIGMYIHNCEKVYFKYFKEDAAYSK 180  
 TQMWEVHVGGQVIVCPTSISSNQISTTETADIQTDNDNRPPQAAAKRRRPADTTDTAQPL 240  
 TKLFCADPALDNRATATNCTNKQRTVCSSNVAPIVHLKGESNSLKLRYRLKPYKELY 300  
 SSMSTTWHWTS DNKNSKNGIVTVTFVTEQQQMFLGTVKIPPTVQISTGFMTL 353

10

HPV33 E5 Accession number W5WL33

1 MIFVFVLCFILFLCLSLLRPLILSISTYAWLLVLVLLWVFGSPLKIFFCYLLFLYLP 60  
 MMCINFHAQHMTQQE 75

15 HPV33 E6 Accession number W6WL33

1 MFQDTEEKPRTLHDLQALETTIHNIELQVCECKKPLQRSEVYDFAFADLTVVYREGNPF 60  
 GICKLCLRFLSKISEYRHYNSVYGNLTLEQTVKKPLNEILIRCIICQRPLCPQEKKRHVD 120  
 LNKRFHNISGRWAGRCAACWRSRRRETAL 149

20 HPV33 E7 Accession number W7WL33

1 MRGHKPTLKEYVLDLYPEPTDLYCYEQLSDSDEDEGLDRPDGQAQPATADYYIVTCCHT 60  
 CNTTVRLCVNSTASDLRTIQQLMGTVNIVCPTCAQQ 97

HPV33 L1 Accession number P1WL33

25 1 MSVWRPSEATVYLPPVPVSKVSTDEYVSRTSIYYIYAGSSRLLAVGHPYFSIKNPTNAKK 60  
 LLVPKVSGLQYRVFRVRLPDPNKGFPDTSFYNPDTQRLVWACVGLGIRGQPLGVGISG 120  
 HPLLNFDDTETGNKYPGQPGADNRECLSMYKQTQLCLLGCKPPTGEHWGKGVACTNAA 180  
 PANDCPLELINTIIEDGDMVDTGFGCMDFKTLQANKSDVPIDICGSTCKYPDYLKMTSE 240  
 PYGDSLFFFLRREQMFVRHFFNRAGTLGEAVPDDLYIKSGTTASIQSSAFFPTPSGSMV 300  
 30 TSESQLENKPYWLQRAQGHNNGICWGNQVFVTVVDTRSTNMTLCTQVTSdstyknENFK 360  
 EYIRHVEEYDLQFVFLCKVTLTAEVMTYIHAMNPDILEDWQFGLTPPPSASLQDTYRFV 420  
 TSQAITCQKTVPPKEKEDPLGKYTFWEVDLKEKFSADLDQFPLGRKFLLQAGLKAKPKLK 480  
 RAAPTSTRTSSAKRKKVKK 499

35 HPV33 L2 Accession number P2WL33

1 MRHKRSTRKRASATQLYQTCKATGTCPPDVIPKVEGSTIADQILKYGSLGVFFGGLGIG 60  
 TGSGSGGRTGYVPIGTDPPATAIPLQPIRPPVTVDTVGPLDSSIVSLIEETSFIAGAPA 120  
 PSIPTPSGFDVTTSDATTPAI INVSSVGESSIQTISTHLNPTFTEPSVLHPPAPAEASGH 180  
 FIFSSPTVSTQSYENIPMDTFVVSTDSNVTSSTPIPGSRPVARLGLYSRNTQQVKVVDP 240  
 40 AFLTSPHKLITYDNPAFESFDPEDTLQFQHSDISPAPDPDFLDIIALHRPAITSRRHTVR 300  
 FSRVGQKATLKTRSGKQIGARIHYQDLSPIVPLDHTVPNEQYELQPLHDTSTSSYSIND 360

GLYDVYADDVDNVHTPMQHSYSTFATTRTSNVSIPLNTGFDTPVMSGPDIPSPLFPTSSP 420  
FVPISPPFFPDTIVVDGADFLHPSYFILRRRRKRFPYFFTDVRVAA 467

HPV56 E2 Accession number S36581

5 1 MVPCLQVCKAKACSAIEVQIALESSTTIYNNEEWTLRDTCEELWLTEPKKCFKKEGQHI 60  
EVWFDGSKNNCMQYVAWKYIYNGDCGWQKVCSGVDYRGIYYVHDGHKTYTDFEQEAKK 120  
FGCKNIWEVHMENESIYCPDSVSSTCRYNVSPVETVNEYNTHKTTTTSTSVGNQDAAVS 180  
HRPGKRPRRLRESEFSSRESHAKCVYTHTHISDTDNDSRSRSINNNNHPGDKTTPVVHL 240  
KGEPNRLKCCRYRFQKYKTLFVDVTSTYHWTSTDNKNYSIITIIYKDETQRNSFLSHVKI 300  
10 PVVYRLVWDK 310

HPV56 E6 Accession number W6WL56

1 MEPQFNPNQERPRSLHHLSEVLEIPLIDLRLSCVYCKKELTRAEVYNFACTELKLVRDD 60  
FPYAVCRVCLLFYSKVRKYRYDYSVYGATLESITKKQLCDLLIRCYRCQSPLTPEEKQL 120  
15 HCDRKRFRFLIAHGWGTGSLGCRQTSREPRESTV 155

HPV56 E7 Accession number S36580

1 MHGKVPTLQDVVLELTPQTEIDLQCNELDSSSEDEDEDEVDHLQERPOQARQAKQHTCYL 60  
IHVPCCECKFVVQLDIQSTKEDLRVVQQLMGALTVTCPLCASN 105  
20

HPV56 L1 Accession number S38563

1 MMLPMYIYRDPPLHYGLCIFLDVGAVNVFPIFLQMATWRPSENKVYLPPTPVSKVVATD 60  
SYVKRTSIFYHAGSSRLAVGHPPYSVTKDNTKTNIPKVSAYQYRVFRVRLPDPNKFGLP 120  
DTNIYNPDQERLVWACVGLVGRGQPLGAGLSGHPLFNRLDDTESSNLANNVIEDSRDN 180  
25 ISVDGKQTQLCIVGCTPAMGEHWTKGAVCKSTQVTTGDCPPLALINTPIEDGDMIDTGFG 240  
AMDFKVLQSSKAEVPLDIVQSTCKYPDYLKMSADAYGDSMWFYLRREQLFARHYFNRAK 300  
VGETIPAELYLKSGNGREPPSSSVYVATPSGSMITSEAQLFNKPYWLQRAQGHNNGICWG 360  
NQLFVTVVDTTSTNMTISTATEQLSKYDARKINQYLRHVEEYELQFVPQLCKITLSAEV 420  
MAYLHNMMANLLEDWNIGLSPPVATSLEDKYRYVRSTAITCQREQPTEKQDPLAKYKFW 480  
30 DVNLQDSFSTDLDQFPLGRKFLMQLGTRSKPAVATSKKRSAPTSTSTPAKRKR 534

HPV56 L2 Accession number S36582

1 MVAHRATRRKRASATQLYKTCKLSGTCPEDVVNKIEQKTWADKILQWGSLEFTYFGGLGIG 60  
TGTGSGGRAGYVPLGSRPSTIVDVTTPARPIVSVESVGPTDPSIVTLVEESSVIESGAGIP 120  
35 NFTGSGGFETSSSTTTTAVLDITPTSSTVHVSSTHITNPLFIDPPVIEAPQTGEVSGNI 180  
LISTPTSGIHSYEEIPMQTFVHSGSGTEPISSTPIPGFRRIAAPRLYRKAQQVKVTDPA 240  
FLDRPATLVADNPLFEGTDTSLAFSPSGVAPDPDFMNIVALHRPAFTTRRGVRFVSRG 300  
RKATIQTTRGTQIGARVHYYYDISPIAQAEIEMQPLLSANNSFDGLYDIYANIDDEAPG 360  
LSSQSVATPSAHLPIKPSTLSFASNTTINVTAPLGNVWETPFYSGPDIVLPTGPSTWPFVP 420  
40 QSPYDVTHDVYIQGSSFALWPVYFFRRRRRKRIPIYFFADGDVAA 464

**HLA Class I Motifs Indicative of CTL Inducing Peptide Epitopes:**

The primary anchor residues of the HLA class I peptide epitope supermotifs and motifs delineated below are summarized in Table I. The HLA class I motifs set out in Table I(a) are those most particularly relevant to the invention claimed here. Primary and secondary anchor positions are summarized in Table II. Allele-specific HLA molecules that comprise HLA class I supertype families are listed in Table VI. In some cases, peptide epitopes may be listed in both a motif and a supermotif Table. The relationship of a particular motif and respective supermotif is indicated in the description of the individual motifs.

### 10 III.D.1. HLA-A1 supermotif

The HLA-A1 supermotif is characterized by the presence in peptide ligands of a small (T or S) or hydrophobic (L, I, V, or M) primary anchor residue in position 2, and an aromatic (Y, F, or W) primary anchor residue at the C-terminal position of the epitope. The corresponding family of HLA molecules that bind to the A1 supermotif (*i.e.*, the HLA-A1 supertype) is comprised of at least A\*0101, A\*2601, A\*2602, A\*2501, and A\*3201 (*see, e.g.*, DiBrino, M. *et al.*, *J. Immunol.* 151:5930, 1993; DiBrino, M. *et al.*, *J. Immunol.* 152:620, 1994; Kondo, A. *et al.*, *Immunogenetics* 45:249, 1997). Other allele-specific HLA molecules predicted to be members of the A1 superfamily are shown in Table VI. Peptides binding to each of the individual HLA proteins can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

20 Representative peptide epitopes that comprise the A1 supermotif are set forth in Table VII.

### III.D.2. HLA-A2 supermotif

25 Primary anchor specificities for allele-specific HLA-A2.1 molecules (*see, e.g.*, Falk *et al.*, *Nature* 351:290-296, 1991; Hunt *et al.*, *Science* 255:1261-1263, 1992; Parker *et al.*, *J. Immunol.* 149:3580-3587, 1992; Ruppert *et al.*, *Cell* 74:929-937, 1993) and cross-reactive binding among HLA-A2 and -A28 molecules have been described. (*See, e.g.*, Fruci *et al.*, *Human Immunol.* 38:187-192, 1993; Tanigaki *et al.*, *Human Immunol.* 39:155-162, 1994; Del Guercio *et al.*, *J. Immunol.* 154:685-693, 1995; Kast *et al.*, *J. Immunol.* 152:3904-3912, 1994 for reviews of relevant data.) These primary anchor residues define the HLA-A2 supermotif; which presence in peptide ligands corresponds to the ability to bind several different HLA-A2 and -A28 molecules. The HLA-A2 supermotif comprises peptide ligands with L, I, V, M, A, T, or Q as a primary anchor residue at position 2 and L, I, V, M, A, or T as a primary anchor residue at the C-terminal position of the epitope.

35 The corresponding family of HLA molecules (*i.e.*, the HLA-A2 supertype that binds these peptides) is comprised of at least: A\*0201, A\*0202, A\*0203, A\*0204, A\*0205, A\*0206, A\*0207, A\*0209, A\*0214, A\*6802, and A\*6901. Other allele-specific HLA molecules predicted to be members of the A2 superfamily are shown in Table VI. As explained in detail below, binding to each of the individual allele-specific HLA molecules can be modulated by substitutions at the primary anchor and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

Representative peptide epitopes that comprise an A2 supermotif are set forth in Table VIII. The motifs comprising the primary anchor residues V, A, T, or Q at position 2 and L, I, V, A, or T at the C-terminal position are those most particularly relevant to the invention claimed herein.

#### 5 III.D.3. HLA-A3 supermotif

The HLA-A3 supermotif is characterized by the presence in peptide ligands of A, L, I, V, M, S, or, T as a primary anchor at position 2, and a positively charged residue, R or K, at the C-terminal position of the epitope, *e.g.*, in position 9 of 9-mers (*see, e.g.*, Sidney *et al.*, *Hum. Immunol.* 45:79, 1996). Exemplary members of the corresponding family of HLA molecules (the HLA-A3 supertype) that bind the  
 10 A3 supermotif include at least A\*0301, A\*1101, A\*3101, A\*3301, and A\*6801. Other allele-specific HLA molecules predicted to be members of the A3 supertype are shown in Table VI. As explained in detail below, peptide binding to each of the individual allele-specific HLA proteins can be modulated by substitutions of amino acids at the primary and/or secondary anchor positions of the peptide, preferably choosing respective residues specified for the supermotif.

15 Representative peptide epitopes that comprise the A3 supermotif are set forth in Table IX.

#### III.D.4. HLA-A24 supermotif

The HLA-A24 supermotif is characterized by the presence in peptide ligands of an aromatic (F, W, or Y) or hydrophobic aliphatic (L, I, V, M, or T) residue as a primary anchor in position 2,  
 20 and Y, F, W, L, I, or M as primary anchor at the C-terminal position of the epitope (*see, e.g.*, Sette and Sidney, *Immunogenetics* 1999 Nov;50(3-4):201-12, Review). The corresponding family of HLA molecules that bind to the A24 supermotif (*i.e.*, the A24 supertype) includes at least A\*2402, A\*3001, and A\*2301. Other allele-specific HLA molecules predicted to be members of the A24 supertype are shown in Table VI. Peptide binding to each of the allele-specific HLA molecules can be modulated by substitutions at primary  
 25 and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

Representative peptide epitopes that comprise the A24 supermotif are set forth in Table X.

#### III.D.5. HLA-B7 supermotif

30 The HLA-B7 supermotif is characterized by peptides bearing proline in position 2 as a primary anchor, and a hydrophobic or aliphatic amino acid (L, I, V, M, A, F, W, or Y) as the primary anchor at the C-terminal position of the epitope. The corresponding family of HLA molecules that bind the B7 supermotif (*i.e.*, the HLA-B7 supertype) is comprised of at least twenty six HLA-B proteins including:  
 B\*0702, B\*0703, B\*0704, B\*0705, B\*1508, B\*3501, B\*3502, B\*3503, B\*3504, B\*3505, B\*3506,  
 35 B\*3507, B\*3508, B\*5101, B\*5102, B\*5103, B\*5104, B\*5105, B\*5301, B\*5401, B\*5501, B\*5502, B\*5601, B\*5602, B\*6701, and B\*7801 (*see, e.g.*, Sidney, *et al.*, *J. Immunol.* 154:247, 1995; Barber, *et al.*, *Curr. Biol.* 5:179, 1995; Hill, *et al.*, *Nature* 360:434, 1992; Rammensee, *et al.*, *Immunogenetics* 41:178, 1995 for reviews of relevant data). Other allele-specific HLA molecules predicted to be members of the B7 supertype are shown in Table VI. As explained in detail below, peptide binding to each of the individual

allele-specific HLA proteins can be modulated by substitutions at the primary and/or secondary anchor positions of the peptide, preferably choosing respective residues specified for the supermotif.

Representative peptide epitopes that comprise the B7 supermotif are set forth in Table XI.

#### 5 III.D.6. HLA-B27 supermotif

The HLA-B27 supermotif is characterized by the presence in peptide ligands of a positively charged (R, H, or K) residue as a primary anchor at position 2, and a hydrophobic (F, Y, L, W, M, I, A, or V) residue as a primary anchor at the C-terminal position of the epitope (*see, e.g., Sidney and Sette, Immunogenetics 1999 Nov;50(3-4):201-12, Review*). Exemplary members of the corresponding family of HLA molecules that bind to the B27 supermotif (*i.e., the B27 supertype*) include at least B\*1401, B\*1402, B\*1509, B\*2702, B\*2703, B\*2704, B\*2705, B\*2706, B\*3801, B\*3901, B\*3902, and B\*7301. Other allele-specific HLA molecules predicted to be members of the B27 supertype are shown in Table VI. Peptide binding to each of the allele-specific HLA molecules can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

15 Representative peptide epitopes that comprise the B27 supermotif are set forth in Table XII.

#### III.D.7. HLA-B44 supermotif

The HLA-B44 supermotif is characterized by the presence in peptide ligands of negatively-charged (D or E) residues as a primary anchor in position 2, and hydrophobic residues (F, W, Y, L, I, M, V, or A) as a primary anchor at the C-terminal position of the epitope (*see, e.g., Sidney et al., Immunol. Today 17:261, 1996*). Exemplary members of the corresponding family of HLA molecules that bind to the B44 supermotif (*i.e., the B44 supertype*) include at least: B\*1801, B\*1802, B\*3701, B\*4001, B\*4002, B\*4006, B\*4402, B\*4403, and B\*4006. Peptide binding to each of the allele-specific HLA molecules can be modulated by substitutions at primary and/or secondary anchor positions; preferably choosing respective residues specified for the supermotif.

#### III.D.8. HLA-B58 supermotif

The HLA-B58 supermotif is characterized by the presence in peptide ligands of a small aliphatic residue (A, S, or T) as a primary anchor residue at position 2, and an aromatic or hydrophobic residue (F, W, Y, L, I, V, M, or A) as a primary anchor residue at the C-terminal position of the epitope (*see, e.g., Sidney and Sette, Immunogenetics 1999 Nov;50(3-4):201-12, Review*). Exemplary members of the corresponding family of HLA molecules that bind to the B58 supermotif (*i.e., the B58 supertype*) include at least: B\*1516, B\*1517, B\*5701, B\*5702, and B\*5801. Other allele-specific HLA molecules predicted to be members of the B58 supertype are shown in Table VI. Peptide binding to each of the allele-specific HLA molecules can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

Representative peptide epitopes that comprise the B58 supermotif are set forth in Table XIII.

40

**III.D.9. HLA-B62 supermotif**

The HLA-B62 supermotif is characterized by the presence in peptide ligands of the polar aliphatic residue Q or a hydrophobic aliphatic residue (L, V, M, I, or P) as a primary anchor in position 2, and a hydrophobic residue (F, W, Y, M, I, V, L, or A) as a primary anchor at the C-terminal position of the epitope (see, e.g., Sidney and Sette, *Immunogenetics* 1999 Nov;50(3-4):201-12, Review). Exemplary members of the corresponding family of HLA molecules that bind to the B62 supermotif (i.e., the B62 supertype) include at least: B\*1501, B\*1502, B\*1513, and B5201. Other allele-specific HLA molecules predicted to be members of the B62 supertype are shown in Table VI. Peptide binding to each of the allele-specific HLA molecules can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

Representative peptide epitopes that comprise the B62 supermotif are set forth in Table XIV.

**III.D.10. HLA-A1 motif**

The HLA-A1 motif is characterized by the presence in peptide ligands of T, S, or M as a primary anchor residue at position 2 and the presence of Y as a primary anchor residue at the C-terminal position of the epitope. An alternative allele-specific A1 motif is characterized by a primary anchor residue at position 3 rather than position 2. This motif is characterized by the presence of D, E, A, or S as a primary anchor residue in position 3, and a Y as a primary anchor residue at the C-terminal position of the epitope (see, e.g., DiBrino *et al.*, *J. Immunol.*; 152:620, 1994; Kondo *et al.*, *Immunogenetics* 45:249, 1997; and Kubo *et al.*, *J. Immunol.* 152:3913, 1994 for reviews of relevant data). Peptide binding to HLA A1 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

Representative peptide epitopes that comprise either A1 motif are set forth in Table XV.

Those epitopes comprising T, S, or M at position 2 and Y at the C-terminal position are also included in the listing of HLA-A1 supermotif-bearing peptides listed in Table VII, as these residues are a subset of the A1 supermotif primary anchors.

**III.D.11. HLA-A\*0201 motif**

An HLA-A2\*0201 motif was determined to be characterized by the presence in peptide ligands of L or M as a primary anchor residue in position 2, and L or V as a primary anchor residue at the C-terminal position of a 9-residue peptide (see, e.g., Falk *et al.*, *Nature* 351:290-296, 1991) and was further found to comprise an I at position 2 and I or A at the C-terminal position of a nine amino acid peptide (see, e.g., Hunt *et al.*, *Science* 255:1261-1263, March 6, 1992; Parker *et al.*, *J. Immunol.* 149:3580-3587, 1992). The A\*0201 allele-specific motif has also been defined by the present inventors to additionally comprise V, A, T, or Q as a primary anchor residue at position 2, and M or T as a primary anchor residue at the C-terminal position of the epitope (see, e.g., Kast *et al.*, *J. Immunol.* 152:3904-3912, 1994). Thus, the HLA-A\*0201 motif comprises peptide ligands with L, I, V, M, A, T, or Q as primary anchor residues at position 2 and L, I, V, M, A, or T as a primary anchor residue at the C-terminal position of the epitope. The preferred and tolerated residues that characterize the primary anchor positions of the HLA-A\*0201 motif



are identical to the residues describing the A2 supermotif. (For reviews of relevant data, *see, e.g.*, Del Guercio *et al.*, *J. Immunol.* 154:685-693, 1995; Ruppert *et al.*, *Cell* 74:929-937, 1993; Sidney *et al.*, *Immunol. Today* 17:261-266, 1996; Sette and Sidney, *Curr. Opin. in Immunol.* 10:478-482, 1998).

5 Secondary anchor residues that characterize the A\*0201 motif have additionally been defined (*see, e.g.*, Ruppert *et al.*, *Cell* 74:929-937, 1993). These are shown in Table II. Peptide binding to HLA-A\*0201 molecules can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

Representative peptide epitopes that comprise an A\*0201 motif are set forth in Table VIII. The A\*0201 motifs comprising the primary anchor residues V, A, T, or Q at position 2 and L, I, V, A, or T at the C-terminal position are those most particularly relevant to the invention claimed herein.

#### III.D.12. HLA-A3 motif

The HLA-A3 motif is characterized by the presence in peptide ligands of L, M, V, I, S, A, T, F, C, G, or D as a primary anchor residue at position 2, and the presence of K, Y, R, H, F, or A as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, DiBrino *et al.*, *Proc. Natl. Acad. Sci USA* 90:1508, 1993; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994). Peptide binding to HLA-A3 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

Representative peptide epitopes that comprise the A3 motif are set forth in Table XVI. Those epitopes that also comprise the A3 supermotif are also listed in Table IX. The A3 supermotif primary anchor residues comprise a subset of the A3- and A11-allele specific motif primary anchor residues.

#### III.D.13. HLA-A11 motif

25 The HLA-A11 motif is characterized by the presence in peptide ligands of V, T, M, L, I, S, A, G, N, C, D, or F as a primary anchor residue in position 2, and K, R, Y, or H as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, Zhang *et al.*, *Proc. Natl. Acad. Sci USA* 90:2217-2221, 1993; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994). Peptide binding to HLA-A11 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

Representative peptide epitopes that comprise the A11 motif are set forth in Table XVII; peptide epitopes comprising the A3 allele-specific motif are also present in this Table because of the extensive overlap between the A3 and A11 motif primary anchor specificities. Further, those peptide epitopes that comprise the A3 supermotif are also listed in Table IX.

#### III.D.14. HLA-A24 motif

The HLA-A24 motif is characterized by the presence in peptide ligands of Y, F, W, or M as a primary anchor residue in position 2, and F, L, I, or W as a primary anchor residue at the C-terminal position of the epitope (*see, e.g.*, Kondo *et al.*, *J. Immunol.* 155:4307-4312, 1995; and Kubo *et al.*, *J. Immunol.* 152:3913-3924, 1994). Peptide binding to HLA-A24 molecules can be modulated by

substitutions at primary and/or secondary anchor positions; preferably choosing respective residues specified for the motif.

Representative peptide epitopes that comprise the A24 motif are set forth in Table XVIII. These epitopes are also listed in Table X, which sets forth HLA-A24-supermotif-bearing peptides, as the primary anchor residues characterizing the A24 allele-specific motif comprise a subset of the A24 supermotif primary anchor residues.

#### Motifs Indicative of Class II HTL Inducing Peptide Epitopes

The primary and secondary anchor residues of the HLA class II peptide epitope supermotifs and motifs delineated below are summarized in Table III.

#### **III.D.15. HLA DR-1-4-7 supermotif**

Motifs have also been identified for peptides that bind to three common HLA class II allele-specific HLA molecules: HLA DRB1\*0401, DRB1\*0101, and DRB1\*0701 (*see, e.g.,* the review by Southwood *et al. J. Immunology* 160:3363-3373, 1998). Collectively, the common residues from these motifs delineate the HLA DR-1-4-7 supermotif. Peptides that bind to these DR molecules carry a supermotif characterized by a large aromatic or hydrophobic residue (Y, F, W, L, I, V, or M) as a primary anchor residue in position 1, and a small, non-charged residue (S, T, C, A, P, V, I, L, or M) as a primary anchor residue in position 6 of a 9-mer core region. Allele-specific secondary effects and secondary anchors for each of these HLA types have also been identified (Southwood *et al., supra*). These are set forth in Table III. Peptide binding to HLA- DRB1\*0401, DRB1\*0101, and/or DRB1\*0701 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the supermotif.

Representative 9-mer epitopes comprising the DR-1-4-7 supermotif, wherein position 1 of the supermotif is at position 1 of the nine-residue core, are set forth in Table XIX. Exemplary epitopes of 15 amino acids in length that comprises the nine residue core include the three residues on either side that flank the nine residue core. HTL epitopes that comprise the core sequences can also be of lengths other than 15 amino acids, *supra*. Accordingly, epitopes of the invention include sequences that typically comprise the nine residue core plus 1, 2, 3 (as in the exemplary 15-mer), 4, or 5 flanking residues on either side of the nine residue core.

#### **III.D.16. HLA DR3 motifs**

Two alternative motifs (*i.e.,* submotifs) characterize peptide epitopes that bind to HLA-DR3 molecules (*see, e.g.,* Geluk *et al., J. Immunol.* 152:5742, 1994). In the first motif (submotif DR3A) a large, hydrophobic residue (L, I, V, M, F, or Y) is present in anchor position 1 of a 9-mer core, and D is present as an anchor at position 4, towards the carboxyl terminus of the epitope. As in other class II motifs, core position 1 may or may not occupy the peptide N-terminal position.

The alternative DR3 submotif provides for lack of the large, hydrophobic residue at anchor position 1, and/or lack of the negatively charged or amide-like anchor residue at position 4, by the presence of a positive charge at position 6 towards the carboxyl terminus of the epitope. Thus, for the

alternative allele-specific DR3 motif (submotif DR3B): L, I, V, M, F, Y, A, or Y is present at anchor position 1; D, N, Q, E, S, or T is present at anchor position 4; and K, R, or H is present at anchor position 6. Peptide binding to HLA-DR3 can be modulated by substitutions at primary and/or secondary anchor positions, preferably choosing respective residues specified for the motif.

5 Representative 9-mer epitopes corresponding to a nine residue sequence comprising the DR3a and DR3b submotifs (wherein position 1 of the motif is at position 1 of the nine residue core) are set forth in Table XXa and b. Exemplary epitopes of 15 amino acids in length that comprises the nine residue core include the three residues on either side that flank the nine residue core. HTL epitopes that comprises the cores sequences can also be of lengths other than 15 amino acids, *supra*. Accordingly, epitopes of the  
10 invention include sequences that typically comprise the nine residue core plus 1, 2, 3 (as in the exemplary 15-mer), 4, or 5 flanking residues on either side of the nine residue core..

Each of the HLA class I or class II epitopes set out in the Tables herein are deemed singly to be an inventive aspect of this application. Further, it is also an inventive aspect of this application that each epitope may be used in combination with any other epitope.

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### III.E. Enhancing Population Coverage of the Vaccine

Vaccines that have broad population coverage are preferred because they are more commercially viable and generally applicable to the most people. Broad population coverage can be obtained using the peptides of the invention (and nucleic acid compositions that encode such peptides)  
20 through selecting peptide epitopes that bind to HLA alleles which, when considered in total, are present in most of the population. The Table below lists the overall frequencies of the HLA class I supertypes in various ethnicities (section a) and the combined population coverage achieved by the A2-, A3-, and B7-supertypes (section b). The A2-, A3-, and B7 supertypes are each present on the average of over 40% in each of these five major ethnic groups. Coverage in excess of 80% is achieved with a combination of these  
25 supermotifs. These results suggest that effective and non-ethnically biased population coverage is achieved upon use of a limited number of cross-reactive peptides. Although the population coverage reached with these three main peptide specificities is high, coverage can be expanded to reach 95% population coverage and above, and more easily achieve truly multispecific responses upon use of additional supermotif or allele-specific motif bearing peptides.

30 The B44-, A1-, and A24-supertypes are each present, on average, in a range from 25% to 40% in these major ethnic populations (section a). While less prevalent overall, the B27-, B58-, and B62 supertypes are each present with a frequency >25% in at least one major ethnic group (section a). In section b, the Table summarizes the estimated prevalence of combinations of HLA supertypes that have been identified in five major ethnic groups. The incremental coverage obtained by the inclusion of A1-, A24-,  
35 and B44-supertypes to the A2, A3, and B7 coverage and coverage obtained with all of the supertypes described herein, is shown.

The data presented herein, together with the previous definition of the A2-, A3-, and B7-supertypes, indicates that all antigens, with the possible exception of A29, B8, and B46, can be classified into a total of nine HLA supertypes. By including epitopes from the six most frequent supertypes, an  
40 average population coverage of 99% is obtained for five major ethnic groups.

## Population coverage with combined HLA Supertypes

HLA-SUPERTYPES	PHENOTYPIC FREQUENCY					
	Caucasian	North American Black	Japanese	Chinese	Hispanic	Average
<b>a. Individual Supertypes</b>						
A2	45.8	39.0	42.4	45.9	43.0	43.2
A3	37.5	42.1	45.8	52.7	43.1	44.2
B7	43.2	55.1	57.1	43.0	49.3	49.5
A1	47.1	16.1	21.8	14.7	26.3	25.2
A24	23.9	38.9	58.6	40.1	38.3	40.0
B44	43.0	21.2	42.9	39.1	39.0	37.0
B27	28.4	26.1	13.3	13.9	35.3	23.4
B62	12.6	4.8	36.5	25.4	11.1	18.1
B58	10.0	25.1	1.6	9.0	5.9	10.3
<b>b. Combined Supertypes</b>						
A2, A3, B7	84.3	86.8	89.5	89.8	86.8	87.4
A2, A3, B7, A24, B44, A1	99.5	98.1	100.0	99.5	99.4	99.3
A2, A3, B7, A24, B44, A1, B27, B62, B58	99.9	99.6	100.0	99.8	99.9	99.8

## III.F. Immune Response-Stimulating Peptide Analogs

- 5 In general, CTL and HTL responses to whole antigens are not directed against all possible epitopes. Rather, they are restricted to a few "immunodominant" determinants (Zinkernagel, *et al.*, *Adv. Immunol.* 27:5159, 1979; Bennink, *et al.*, *J. Exp. Med.* 168:1935-1939, 1988; Rawle, *et al.*, *J. Immunol.* 146:3977-3984, 1991). It has been recognized that immunodominance (Benacerraf, *et al.*, *Science* 175:273-279, 1972) could be explained by either the ability of a given epitope to selectively bind a particular HLA
- 10 protein (determinant selection theory) (Vitiello, *et al.*, *J. Immunol.* 131:1635, 1983); Rosenthal, *et al.*, *Nature* 267:156-158, 1977), or to be selectively recognized by the existing TCR (T cell receptor) specificities (repertoire theory) (Klein, J., *IMMUNOLOGY, THE SCIENCE OF SELF/NONSELF DISCRIMINATION*, John Wiley & Sons, New York, pp. 270-310, 1982). It has been demonstrated that additional factors, mostly linked to processing events, can also play a key role in dictating, beyond strict immunogenicity,
- 15 which of the many potential determinants will be presented as immunodominant (Sercarz, *et al.*, *Annu. Rev. Immunol.* 11:729-766, 1993).

- The concept of dominance and subdominance is relevant to immunotherapy of both infectious diseases and cancer. For example, in the course of chronic viral disease, recruitment of subdominant epitopes can be important for successful clearance of the infection, especially if dominant
- 20 CTL or HTL specificities have been inactivated by functional tolerance, suppression, mutation of viruses and other mechanisms (Franco, *et al.*, *Curr. Opin. Immunol.* 7:524-531, 1995). In the case of cancer and tumor antigens, CTLs recognizing at least some of the highest binding affinity peptides might be functionally inactivated. Lower binding affinity peptides are preferentially recognized at these times, and may therefore be preferred in therapeutic or prophylactic anti-cancer vaccines.

- 25 In particular, it has been noted that a significant number of epitopes derived from known non-viral tumor associated antigens (TAA) bind HLA class I with intermediate affinity (IC<sub>50</sub> in the 50-500

nM range). For example, it has been found that 8 of 15 known TAA peptides recognized by tumor infiltrating lymphocytes (TIL) or CTL bound in the 50-500 nM range. (These data are in contrast with estimates that 90% of known viral antigens were bound by HLA class I molecules with  $IC_{50}$  of 50 nM or less, while only approximately 10% bound in the 50-500 nM range (Sette, *et al.*, *J. Immunol.*, 153:558-5592, 1994). In the cancer setting this phenomenon is probably due to elimination or functional inhibition of the CTL recognizing several of the highest binding peptides, presumably because of T cell tolerization events.

Without intending to be bound by theory, it is believed that because T cells to dominant epitopes may have been clonally deleted, selecting subdominant epitopes may allow existing T cells to be recruited, which will then lead to a therapeutic or prophylactic response. However, the binding of HLA molecules to subdominant epitopes is often less vigorous than to dominant ones. Accordingly, there is a need to be able to modulate the binding affinity of particular immunogenic epitopes for one or more HLA molecules, and thereby to modulate the immune response elicited by the peptide, for example to prepare analog peptides which elicit a more vigorous response. This ability would greatly enhance the usefulness of peptide epitope-based vaccines and therapeutic agents.

Although peptides with suitable cross-reactivity among all alleles of a superfamily are identified by the screening procedures described above, cross-reactivity is not always as complete as possible, and in certain cases procedures to increase cross-reactivity of peptides can be useful; moreover, such procedures can also be used to modify other properties of the peptides such as binding affinity or peptide stability. Having established the general rules that govern cross-reactivity of peptides for HLA alleles within a given motif or supermotif, modification (*i.e.*, analoging) of the structure of peptides of particular interest in order to achieve broader (or otherwise modified) HLA binding capacity can be performed. More specifically, peptides which exhibit the broadest cross-reactivity patterns, can be produced in accordance with the teachings herein. The present concepts related to analog generation are set forth in greater detail in co-pending U.S.S.N. 09/226,775 filed 1/6/99.

In brief, the strategy employed utilizes the motifs or supermotifs which correlate with binding to certain HLA molecules. The motifs or supermotifs are defined by having primary anchors, and in many cases secondary anchors. Analog peptides can be created by substituting amino acid residues at primary anchor, secondary anchor, or at primary and secondary anchor positions. Generally, analogs are made for peptides that already bear a motif or supermotif. Preferred secondary anchor residues of supermotifs and motifs that have been defined for HLA class I and class II binding peptides are shown in Tables II and III, respectively.

For a number of the motifs or supermotifs in accordance with the invention, residues are defined which are deleterious to binding to allele-specific HLA molecules or members of HLA supertypes that bind the respective motif or supermotif (Tables II and III). Accordingly, removal of such residues that are detrimental to binding can be performed in accordance with the present invention. For example, in the case of the A3 supertype, when all peptides that have such deleterious residues are removed from the population of peptides used in the analysis, the incidence of cross-reactivity increased from 22% to 37% (*see, e.g.*, Sidney, J. *et al.*, *Hu. Immunol.* 45:79, 1996). Thus, one strategy to improve the cross-reactivity of peptides within a given supermotif is simply to delete one or more of the deleterious residues present

within a peptide and substitute a small "neutral" residue such as Ala (that may not influence T cell recognition of the peptide). An enhanced likelihood of cross-reactivity is expected if, together with elimination of detrimental residues within a peptide, "preferred" residues associated with high affinity binding to an allele-specific HLA molecule or to multiple HLA molecules within a superfamily are inserted.

5 To ensure that an analog peptide, when used as a vaccine, actually elicits a CTL response to the native epitope *in vivo* (or, in the case of class II epitopes, elicits helper T cells that cross-react with the wild type peptides), the analog peptide may be used to immunize T cells *in vitro* from individuals of the appropriate HLA allele. Thereafter, the immunized cells' capacity to induce lysis of wild type peptide sensitized target cells is evaluated. It will be desirable to use as antigen presenting cells, cells that have  
10 been either infected, or transfected with the appropriate genes, or, in the case of class II epitopes only, cells that have been pulsed with whole protein antigens, to establish whether endogenously produced antigen is also recognized by the relevant T cells.

Another embodiment of the invention is to create analogs of weak binding peptides, to thereby ensure adequate numbers of cross-reactive cellular binders. Class I binding peptides exhibiting  
15 binding affinities of 500-5000 nM, and carrying an acceptable but suboptimal primary anchor residue at one or both positions can be "fixed" by substituting preferred anchor residues in accordance with the respective supertype. The analog peptides can then be tested for crossbinding activity.

Another embodiment for generating effective peptide analogs involves the substitution of residues that have an adverse impact on peptide stability or solubility in, *e.g.*, a liquid environment. This  
20 substitution may occur at any position of the peptide epitope. For example, a cysteine (C) can be substituted out in favor of  $\alpha$ -amino butyric acid. Due to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally so as to reduce binding capacity. Substituting  $\alpha$ -amino butyric acid for C not only alleviates this problem, but actually improves binding and crossbinding capability in certain instances (*see, e.g.*, the review by Sette *et al.*, In: Persistent Viral  
25 Infections, Eds. R. Ahmed and I. Chen, John Wiley & Sons, England, 1999). Substitution of cysteine with  $\alpha$ -amino butyric acid may occur at any residue of a peptide epitope, *i.e.* at either anchor or non-anchor positions.

### 30 III.G. Computer Screening of Protein Sequences from Disease-Related Antigens for Supermotif- or Motif-Bearing Peptides

In order to identify supermotif- or motif-bearing epitopes in a target antigen, a native protein sequence, *e.g.*, a tumor-associated antigen, or sequences from an infectious organism, or a donor tissue for transplantation, is screened using a means for computing, such as an intellectual calculation or a computer, to determine the presence of a supermotif or motif within the sequence. The information  
35 obtained from the analysis of native peptide can be used directly to evaluate the status of the native peptide or may be utilized subsequently to generate the peptide epitope.

Computer programs that allow the rapid screening of protein sequences for the occurrence of the subject supermotifs or motifs are encompassed by the present invention; as are programs that permit the generation of analog peptides. These programs are implemented to analyze any identified amino acid  
40 sequence or operate on an unknown sequence and simultaneously determine the sequence and identify

motif-bearing epitopes thereof; analogs can be simultaneously determined as well. Generally, the identified sequences will be from a pathogenic organism or a tumor-associated peptide. For example, the target molecules considered herein include, without limitation, the E1, E2, E4, E5a, E5b, E6, E7, L1 and L2 proteins of HPV.

In cases where the sequences of multiple variants of the same target protein are available, potential peptide epitopes can also be selected on the basis of their conservancy. For example, a criterion for conservancy may define that the entire sequence of an HLA class I binding peptide or the entire 9-mer core of a class II binding peptide, be conserved in a designated percentage, of the sequences evaluated for a specific protein antigen.

To target a broad population that may be infected with a number of different strains, it is preferable to include in vaccine compositions epitopes that are representative of HPV antigen sequences from different HPV strains. As appreciated by those in the art, regions with greater or lesser degrees of conservancy among HPV strains can be employed as appropriate for a given antigenic target.

It is important that the selection criteria utilized for prediction of peptide binding are as accurate as possible, to correlate most efficiently with actual binding. Prediction of peptides that bind, for example, to HLA-A\*0201, on the basis of the presence of the appropriate primary anchors, is positive at about a 30% rate (*see, e.g.,* Ruppert, J. *et al. Cell* 74:929, 1993). However, by extensively analyzing peptide-HLA binding data disclosed herein, data in related patent applications, and data in the art, the present inventors have developed a number of allele-specific polynomial algorithms that dramatically increase the predictive value over identification on the basis of the presence of primary anchor residues alone. These algorithms take into account not only the presence or absence of primary anchors, but also consider the positive or deleterious presence of secondary anchor residues (to account for the impact of different amino acids at different positions). The algorithms are essentially based on the premise that the overall affinity (or  $\Delta G$ ) of peptide-HLA interactions can be approximated as a linear polynomial function of the type:

$$\Delta G = a_{1i} \times a_{2j} \times a_{3k} \dots \times a_{ni}$$

where  $a_{ji}$  is a coefficient that represents the effect of the presence of a given amino acid ( $j$ ) at a given position ( $i$ ) along the sequence of a peptide of  $n$  amino acids. An important assumption of this method is that the effects at each position are essentially independent of each other. This assumption is justified by studies that demonstrated that peptides are bound to HLA molecules and recognized by T cells in essentially an extended conformation. Derivation of specific algorithm coefficients has been described, for example, in Gulukota, K. *et al., J. Mol. Biol.* 267:1258, 1997.

Additional methods to identify preferred peptide sequences, which also make use of specific motifs, include the use of neural networks and molecular modeling programs (*see, e.g.,* Milik *et al., Nature Biotechnology* 16:753, 1998; Altuvia *et al., Hum. Immunol.* 58:1, 1997; Altuvia *et al., J. Mol. Biol.* 249:244, 1995; Buus, S. *Curr. Opin. Immunol.* 11:209-213, 1999; Brusic, V. *et al., Bioinformatics* 14:121-130, 1998; Parker *et al., J. Immunol.* 152:163, 1993; Meister *et al., Vaccine* 13:581, 1995; Hammer *et al., J. Exp. Med.* 180:2353, 1994; Sturniolo *et al., Nature Biotechnol.* 17:555 1999).

For example, it has been shown that in sets of A\*0201 motif-bearing peptides containing at least one preferred secondary anchor residue while avoiding the presence of any deleterious secondary

anchor residues, 69% of the peptides will bind A\*0201 with an IC<sub>50</sub> less than 500 nM (Ruppert, J. *et al. Cell* 74:929, 1993). These algorithms are also flexible in that cut-off scores may be adjusted to select sets of peptides with greater or lower predicted binding properties, as desired.

In utilizing computer screening to identify peptide epitopes, a protein sequence or translated sequence may be analyzed using software developed to search for motifs, for example the "FINDPATTERNS" program (Devereux, *et al. Nucl. Acids Res.* 12:387-395, 1984) or MotifSearch 1.4 software program (D. Brown, San Diego, CA) to identify potential peptide sequences containing appropriate HLA binding motifs. The identified peptides can be scored using customized polynomial algorithms to predict their capacity to bind specific HLA class I or class II alleles. As appreciated by one of ordinary skill in the art, a large array of computer programming software and hardware options are available in the relevant art which can be employed to implement the motifs of the invention in order to evaluate (e.g., without limitation, to identify epitopes, identify epitope concentration per peptide length, or to generate analogs) known or unknown peptide sequences.

In accordance with the procedures described above, HPV peptide epitopes that are able to bind HLA supertype groups or allele-specific HLA molecules have been identified (Tables VII-XX).

### III.H. Preparation of Peptide Epitopes

Peptides in accordance with the invention can be prepared synthetically, by recombinant DNA technology or chemical synthesis, or from natural sources such as native tumors or pathogenic organisms. Peptide epitopes may be synthesized individually or as polypeptidic peptides. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides may be synthetically conjugated to native fragments or particles.

The peptides in accordance with the invention can be a variety of lengths, and either in their neutral (uncharged) forms or in forms which are salts. The peptides in accordance with the invention are either free of modifications such as glycosylation, side chain oxidation, or phosphorylation; or they contain these modifications, subject to the condition that modifications do not destroy the biological activity of the peptides as described herein.

When possible, it may be desirable to optimize HLA class I binding epitopes of the invention, such as can be used in a polypeptidic construct, to a length of about 8 to about 13 amino acid residues, often 8 to 11, preferably 9 to 10. HLA class II binding peptide epitopes of the invention may be optimized to a length of about 6 to about 30 amino acids in length, preferably to between about 13 and about 20 residues. Preferably, the peptide epitopes are commensurate in size with endogenously processed pathogen-derived peptides or tumor cell peptides that are bound to the relevant HLA molecules, however, the identification and preparation of peptides that comprise epitopes of the invention can also be carried out using the techniques described herein.

In alternative embodiments, epitopes of the invention can be linked as a polypeptidic peptide, or as a minigene that encodes a polypeptidic peptide.

In another embodiment, it is preferred to identify native peptide regions that contain a high concentration of class I and/or class II epitopes. Such a sequence is generally selected on the basis that it contains the greatest number of epitopes per amino acid length. It is to be appreciated that epitopes can



be present in a nested or overlapping manner, e.g. a 10 amino acid long peptide could contain two 9 amino acid long epitopes and one 10 amino acid long epitope; upon intracellular processing, each epitope can be exposed and bound by an HLA molecule upon administration of such a peptide. This larger, preferably multi-epitopic, peptide can be generated synthetically, recombinantly, or via cleavage from the native source.

The peptides of the invention can be prepared in a wide variety of ways. For the preferred relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. (See, for example, Stewart & Young, SOLID PHASE PEPTIDE SYNTHESIS, 2D. ED., Pierce Chemical Co., 1984). Further, individual peptide epitopes can be joined using chemical ligation to produce larger peptides that are still within the bounds of the invention.

Alternatively, recombinant DNA technology can be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989). Thus, recombinant polypeptides which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

The nucleotide coding sequence for peptide epitopes of the preferred lengths contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, *J. Am. Chem. Soc.* 103:3185 (1981). Peptide analogs can be made simply by substituting the appropriate and desired nucleic acid base(s) for those that encode the native peptide sequence; exemplary nucleic acid substitutions are those that encode an amino acid defined by the motifs/super motifs herein. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast, insect or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

### III.I. Assays to Detect T-Cell Responses

Once HLA binding peptides are identified, they can be tested for the ability to elicit a T-cell response. The preparation and evaluation of motif-bearing peptides are described in PCT publications WO 94/20127 and WO 94/03205. Briefly, peptides comprising epitopes from a particular antigen are synthesized and tested for their ability to bind to the appropriate HLA proteins. These assays may involve evaluating the binding of a peptide of the invention to purified HLA class I molecules in relation to the binding of a radioiodinated reference peptide. Alternatively, cells expressing empty class I molecules (*i.e.*

lacking peptide therein) may be evaluated for peptide binding by immunofluorescent staining and flow microfluorimetry. Other assays that may be used to evaluate peptide binding include peptide-dependent class I assembly assays and/or the inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule, typically with an affinity of 500 nM or less, are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary *in vitro* or *in vivo* CTL responses that can give rise to CTL populations capable of reacting with selected target cells associated with a disease.

Analogous assays are used for evaluation of HLA class II binding peptides. HLA class II motif-bearing peptides that are shown to bind, typically at an affinity of 1000 nM or less, are further evaluated for the ability to stimulate HTL responses.

Conventional assays utilized to detect T cell responses include proliferation assays, lymphokine secretion assays, direct cytotoxicity assays, and limiting dilution assays. For example, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells. Alternatively, mutant non-human mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides and that have been transfected with the appropriate human class I gene, may be used to test for the capacity of the peptide to induce *in vitro* primary CTL responses.

Peripheral blood mononuclear cells (PBMCs) may be used as the responder cell source of CTL precursors. The appropriate antigen-presenting cells are incubated with peptide, after which the peptide-loaded antigen-presenting cells are then incubated with the responder cell population under optimized culture conditions. Positive CTL activation can be determined by assaying the culture for the presence of CTLs that kill radio-labeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed forms of the antigen from which the peptide sequence was derived.

Additionally, a method has been devised which allows direct quantification of antigen-specific T cells by staining with Fluorescein-labelled HLA tetrameric complexes (Altman, J. D. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10330, 1993; Altman, J. D. *et al.*, *Science* 274:94, 1996). Other relatively recent technical developments include staining for intracellular lymphokines, and interferon release assays or ELISPOT assays. Tetramer staining, intracellular lymphokine staining and ELISPOT assays all appear to be at least 10-fold more sensitive than more conventional assays (Lalvani, A. *et al.*, *J. Exp. Med.* 186:859, 1997; Dunbar, P. R. *et al.*, *Curr. Biol.* 8:413, 1998; Murali-Krishna, K. *et al.*, *Immunity* 8:177, 1998).

HTL activation may also be assessed using such techniques known to those in the art such as T cell proliferation and secretion of lymphokines, *e.g.* IL-2 (*see, e.g.* Alexander *et al.*, *Immunity* 1:751-761, 1994).

Alternatively, immunization of HLA transgenic mice can be used to determine immunogenicity of peptide epitopes. Several transgenic mouse models including mice with human A2.1, A11 (which can additionally be used to analyze HLA-A3 epitopes), and B7 alleles have been characterized and others (*e.g.*, transgenic mice for HLA-A1 and A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed. Additional transgenic mouse models with other HLA alleles may

be generated as necessary. Mice may be immunized with peptides emulsified in Incomplete Freund's Adjuvant and the resulting T cells tested for their capacity to recognize peptide-pulsed target cells and target cells transfected with appropriate genes. CTL responses may be analyzed using cytotoxicity assays described above. Similarly, HTL responses may be analyzed using such assays as T cell proliferation or secretion of lymphokines.

### III.J. Use of Peptide Epitopes as Diagnostic Agents and for Evaluating Immune Responses

In one aspect of the invention, HLA class I and class II binding peptides as described herein can be used as reagents to evaluate an immune response. The immune response to be evaluated is induced by using as an immunogen any agent that may result in the production of antigen-specific CTLs or HTLs that recognize and bind to the peptide epitope(s) to be employed as the reagent. The peptide reagent need not be used as the immunogen. Assay systems that are used for such an analysis include relatively recent technical developments such as tetramers, staining for intracellular lymphokines and interferon release assays, or ELISPOT assays.

For example, a peptide of the invention is used in a tetramer staining assay to assess peripheral blood mononuclear cells for the presence of antigen-specific CTLs following exposure to a pathogen or immunogen. The HLA-tetrameric complex is used to directly visualize antigen-specific CTLs (see, e.g., Ogg *et al.*, *Science* 279:2103-2106, 1998; and Altman *et al.*, *Science* 174:94-96, 1996) and determine the frequency of the antigen-specific CTL population in a sample of peripheral blood mononuclear cells.

A tetramer reagent using a peptide of the invention is generated as follows: A peptide that binds to an HLA molecule is refolded in the presence of the corresponding HLA heavy chain and  $\beta_2$ -microglobulin to generate a trimolecular complex. The complex is biotinylated at the carboxyl terminal end of the heavy chain at a site that was previously engineered into the protein. Tetramer formation is then induced by the addition of streptavidin. By means of fluorescently labeled streptavidin, the tetramer can be used to stain antigen-specific cells. The cells can then be readily identified, for example, by flow cytometry. Such procedures are used for diagnostic or prognostic purposes. Cells identified by the procedure can also be used for therapeutic purposes.

Peptides of the invention are also used as reagents to evaluate immune recall responses. (see, e.g., Bertoni *et al.*, *J. Clin. Invest.* 100:503-513, 1997 and Penna *et al.*, *J. Exp. Med.* 174:1565-1570, 1991.) For example, patient PBMC samples from individuals infected with HPV are analyzed for the presence of antigen-specific CTLs or HTLs using specific peptides. A blood sample containing mononuclear cells may be evaluated by cultivating the PBMCs and stimulating the cells with a peptide of the invention. After an appropriate cultivation period, the expanded cell population may be analyzed, for example, for CTL or for HTL activity.

The peptides are also used as reagents to evaluate the efficacy of a vaccine. PBMCs obtained from a patient vaccinated with an immunogen are analyzed using, for example, either of the methods described above. The patient is HLA typed, and peptide epitope reagents that recognize the allele-specific molecules present in that patient are selected for the analysis. The immunogenicity of the vaccine is indicated by the presence of HPV epitope-specific CTLs and/or HTLs in the PBMC sample.

The peptides of the invention are also be used to make antibodies, using techniques well known in the art (see, e.g. *CURRENT PROTOCOLS IN IMMUNOLOGY*, Wiley/Greene, NY; and *Antibodies A Laboratory Manual* Harlow, Harlow and Lane, Cold Spring Harbor Laboratory Press, 1989), which may be useful as reagents to diagnose HPV infection. Such antibodies include those that recognize a peptide in the context of an HLA molecule, i.e., antibodies that bind to a peptide-MHC complex.

### III.K. Vaccine Compositions

Vaccines and methods of preparing vaccines that contain an immunogenically effective amount of one or more peptides as described herein are further embodiments of the invention. Once appropriately immunogenic epitopes have been defined, they can be sorted and delivered by various means, herein referred to as "vaccine" compositions. Such vaccine compositions can include, for example, lipopeptides (e.g., Vitiello, A. et al., *J. Clin. Invest.* 95:341, 1995), peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres (see, e.g., Eldridge, et al., *Molec. Immunol.* 28:287-294, 1991; Alonso et al., *Vaccine* 12:299-306, 1994; Jones et al., *Vaccine* 13:675-681, 1995), peptide compositions contained in immune stimulating complexes (ISCOMS) (see, e.g., Takahashi et al., *Nature* 344:873-875, 1990; Hu et al., *Clin Exp Immunol.* 113:235-243, 1998), multiple antigen peptide systems (MAPs) (see e.g., Tam, J. P., *Proc. Natl. Acad. Sci. U.S.A.* 85:5409-5413, 1988; Tam, J.P., *J. Immunol. Methods* 196:17-32, 1996), peptides formulated as multivalent peptides; peptides for use in ballistic delivery systems, typically crystallized peptides, viral delivery vectors (Perkus, M. E. et al., In: *Concepts in vaccine development*, Kaufmann, S. H. E., ed., p. 379, 1996; Chakrabarti, S. et al., *Nature* 320:535, 1986; Hu, S. L. et al., *Nature* 320:537, 1986; Kieny, M.-P. et al., *AIDS Bio/Technology* 4:790, 1986; Top, F. H. et al., *J. Infect. Dis.* 124:148, 1971; Chanda, P. K. et al., *Virology* 175:535, 1990), particles of viral or synthetic origin (e.g., Kofler, N. et al., *J. Immunol. Methods* 192:25, 1996; Eldridge, J. H. et al., *Sem. Hematol.* 30:16, 1993; Falo, L. D., Jr. et al., *Nature Med.* 7:649, 1995), adjuvants (Warren, H. S., Vogel, F. R., and Chedid, L. A. *Annu. Rev. Immunol.* 4:369, 1986; Gupta, R. K. et al., *Vaccine* 11:293, 1993), liposomes (Reddy, R. et al., *J. Immunol.* 148:1585, 1992; Rock, K. L., *Immunol. Today* 17:131, 1996), or, naked or particle absorbed cDNA (Ulmer, J. B. et al., *Science* 259:1745, 1993; Robinson, H. L., Hunt, L. A., and Webster, R. G., *Vaccine* 11:957, 1993; Shiver, J. W. et al., In: *Concepts in vaccine development*, Kaufmann, S. H. E., ed., p. 423, 1996; Cease, K. B., and Berzofsky, J. A., *Annu. Rev. Immunol.* 12:923, 1994 and Eldridge, J. H. et al., *Sem. Hematol.* 30:16, 1993). Toxin-targeted delivery technologies, also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) may also be used.

Vaccine compositions of the invention include nucleic acid-mediated modalities. DNA or RNA encoding one or more of the peptides of the invention can also be administered to a patient. This approach is described, for instance, in Wolff et al., *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; WO 98/04720; and in more detail below. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivacaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery (see, e.g., U.S. Patent No. 5,922,687).

For therapeutic or prophylactic immunization purposes, the peptides of the invention can be expressed by viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus, for example, as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL and/or HTL response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al.*, *Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g. adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella typhi* vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein.

Furthermore, vaccines in accordance with the invention encompass compositions of one or more of the claimed peptides. A peptide can be present in a vaccine individually. Alternatively, the peptide can exist as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides. Polymers have the advantage of increased immunological reaction and, where different peptide epitopes are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the pathogenic organism or tumor-related peptide targeted for an immune response. The composition can be a naturally occurring region of an antigen or can be prepared, e.g., recombinantly or by chemical synthesis.

Carriers that can be used with vaccines of the invention are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like. The vaccines can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, preferably phosphate buffered saline. The vaccines also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are examples of materials well known in the art. Additionally, as disclosed herein, CTL responses can be primed by conjugating peptides of the invention to lipids, such as tripalmitoyl-S-glycerylcysteinylserine (P<sub>3</sub>CSS).

Upon immunization with a peptide composition in accordance with the invention, via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, the immune system of the host responds to the vaccine by producing large amounts of CTLs and/or HTLs specific for the desired antigen. Consequently, the host becomes at least partially immune to later infection, or at least partially resistant to developing an ongoing chronic infection, or derives at least some therapeutic benefit when the antigen was tumor-associated.

In some embodiments, it may be desirable to combine the class I peptide components with components that induce or facilitate neutralizing antibody and/or helper T cell responses to the target antigen of interest. A preferred embodiment of such a composition comprises class I and class II epitopes in accordance with the invention. An alternative embodiment of such a composition comprises a class I and/or class II epitope in accordance with the invention, along with a cross reactive HTL epitope such as PADRE™ (Epimmune, San Diego, CA) molecule (described *e.g.*, in U.S. Patent Number 5,736,142).

A vaccine of the invention can also include antigen-presenting cells (APC), such as dendritic cells (DC), as a vehicle to present peptides of the invention. Vaccine compositions can be created *in vitro*, following dendritic cell mobilization and harvesting, whereby loading of dendritic cells occurs *in vitro*. For example, dendritic cells are transfected, *e.g.*, with a minigene in accordance with the invention, or are pulsed with peptides. The dendritic cell can then be administered to a patient to elicit immune responses *in vivo*.

Vaccine compositions, either DNA- or peptide-based, can also be administered *in vivo* in combination with dendritic cell mobilization whereby loading of dendritic cells occurs *in vivo*.

Antigenic peptides are used to elicit a CTL and/or HTL response *ex vivo*, as well. The resulting CTL or HTL cells, can be used to treat chronic infections, or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a therapeutic vaccine peptide or nucleic acid in accordance with the invention. *Ex vivo* CTL or HTL responses to a particular antigen (infectious or tumor-associated antigen) are induced by incubating in tissue culture the patient's, or genetically compatible, CTL or HTL precursor cells together with a source of antigen-presenting cells (APC), such as dendritic cells, and the appropriate immunogenic peptide. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded into effector cells, the cells are infused back into the patient, where they will destroy (CTL) or facilitate destruction (HTL) of their specific target cell (an infected cell or a tumor cell). Transfected dendritic cells may also be used as antigen presenting cells.

The vaccine compositions of the invention may also be used in combination with other procedures to remove warts or treat HPV infections. Such procedures include cryosurgery, application of caustic agents, electrodesiccation, surgical excision and laser ablation (Fauci et al. HARRISON'S PRINCIPLES OF INTERNAL MEDICINE, 14th ED., McGraw-Hill Co., Inc, 1998), as well as treatment with antiviral drugs such as interferon- $\alpha$  (see, *e.g.*, Stellato, G., et al., Clin. Diagn. Virol. 7(3):167-72 (1997)) or interferon-inducing drugs such as imiquimod. Topical antimetabolites such as 5-fluorouracil may also be applied.

In patients with HPV-associated cancer, the vaccine compositions of the invention can also be used in conjunction with other treatments used for cancer, *e.g.*, surgery, chemotherapy, drug therapies, radiation therapies, *etc.* including use in combination with immune adjuvants such as IL-2, IL-12, GM-CSF, and the like.

Preferably, the following principles are utilized when selecting an array of epitopes for inclusion in a polypeptidic composition for use in a vaccine, or for selecting discrete epitopes to be included in a vaccine and/or to be encoded by nucleic acids such as a minigene. It is preferred that each of the following principles are balanced in order to make the selection. The multiple epitopes to be incorporated in a given vaccine composition may be, but need not be, contiguous in sequence in the native antigen from which the epitopes are derived.

1.) Epitopes are selected which, upon administration, mimic immune responses that have been observed to be correlated with clearance of HPV infection or tumor clearance. For HLA Class I this includes 3-4 epitopes that come from at least one TAA. For HLA Class II a similar rationale is employed; again 3-4 epitopes are selected from at least one TAA (see, *e.g.*, Rosenberg *et al.*, Science

278:1447-1450). Epitopes from one TAA may be used in combination with epitopes from one or more additional TAAs to produce a vaccine that targets tumors with varying expression patterns of frequently-expressed TAAs as described, *e.g.*, in Example 15.

2.) Epitopes are selected that have the requisite binding affinity established to be  
5 correlated with immunogenicity: for HLA Class I an  $IC_{50}$  of 500 nM or less, often 200 nM or less; and for Class II an  $IC_{50}$  of 1000 nM or less.

3.) Sufficient supermotif bearing-peptides, or a sufficient array of allele-specific motif-bearing peptides, are selected to give broad population coverage. For example, it is preferable to have at least 80% population coverage. A Monte Carlo analysis, a statistical evaluation known in the art,  
10 can be employed to assess the breadth, or redundancy of, population coverage.

4.) When selecting epitopes from cancer-related antigens it is often useful to select analogs because the patient may have developed tolerance to the native epitope. When selecting epitopes for infectious disease-related antigens it is preferable to select either native or analoged epitopes.

5.) Of particular relevance are epitopes referred to as "nested epitopes." Nested  
15 epitopes occur where at least two epitopes overlap in a given peptide sequence. A nested peptide sequence can comprise both HLA class I and HLA class II epitopes. When providing nested epitopes, a general objective is to provide the greatest number of epitopes per sequence. Thus, an aspect is to avoid providing a peptide that is any longer than the amino terminus of the amino terminal epitope and the carboxyl terminus of the carboxyl terminal epitope in the peptide. When providing a multi-epitopic sequence, such  
20 as a sequence comprising nested epitopes, it is generally important to screen the sequence in order to insure that it does not have pathological or other deleterious biological properties.

6.) If a polyepitopic protein is created, or when creating a minigene, an objective is to generate the smallest peptide that encompasses the epitopes of interest. This principle is similar, if not the same as that employed when selecting a peptide comprising nested epitopes. However, with an artificial  
25 polyepitopic peptide, the size minimization objective is balanced against the need to integrate any spacer sequences between epitopes in the polyepitopic protein. Spacer amino acid residues can, for example, be introduced to avoid junctional epitopes (an epitope recognized by the immune system, not present in the target antigen, and only created by the man-made juxtaposition of epitopes), or to facilitate cleavage between epitopes and thereby enhance epitope presentation. Junctional epitopes are generally to be avoided  
30 because the recipient may generate an immune response to that non-native epitope. Of particular concern is a junctional epitope that is a "dominant epitope." A dominant epitope may lead to such a zealous response that immune responses to other epitopes are diminished or suppressed.

7.) In cases where the sequences of multiple variants of the same target protein are available, potential peptide epitopes can also be selected on the basis of their conservancy. For example, a  
35 criterion for conservancy may define that the entire sequence of an HLA class I binding peptide or the entire 9-mer core of a class II binding peptide be conserved in a designated percentage of the sequences evaluated for a specific protein antigen.

8.) When selecting an array of epitopes of an infectious agent, it is preferred that at least some of the epitopes are derived from early and late proteins. The early proteins of HPV are  
40 expressed when the virus is replicating, either following acute or dormant infection. Therefore, it is

particularly preferred to use epitopes from early stage proteins to alleviate disease manifestations at the earliest stage possible.

### III.K.1. Minigene Vaccines

5 A number of different approaches are available which allow simultaneous delivery of multiple epitopes. Nucleic acids encoding the peptides of the invention are a particularly useful embodiment of the invention. Epitopes for inclusion in a minigene are preferably selected according to the guidelines set forth in the previous section. A preferred means of administering nucleic acids encoding the peptides of the invention uses minigene constructs encoding a peptide comprising one or multiple epitopes  
10 of the invention.

The use of multi-epitope minigenes is described below and in, e.g., co-pending application U.S.S.N. 09/311,784; Ishioka *et al.*, *J. Immunol.* 162:3915-3925, 1999; An, L. and Whitton, J. L., *J. Virol.* 71:2292, 1997; Thomson, S. A. *et al.*, *J. Immunol.* 157:822, 1996; Whitton, J. L. *et al.*, *J. Virol.* 67:348, 1993; Hanke, R. *et al.*, *Vaccine* 16:426, 1998. For example, a multi-epitope DNA plasmid  
15 encoding supermotif- and/or motif-bearing epitopes derived from multiple regions of one or more HPV antigens, the PADRE universal helper T cell epitope (or multiple HTL epitopes from HPV antigens), and an endoplasmic reticulum-translocating signal sequence can be engineered. A vaccine may also comprise epitopes that are derived from other TAAs.

The immunogenicity of a multi-epitopic minigene can be tested in transgenic mice to  
20 evaluate the magnitude of CTL induction responses against the epitopes tested. Further, the immunogenicity of DNA-encoded epitopes *in vivo* can be correlated with the *in vitro* responses of specific CTL lines against target cells transfected with the DNA plasmid. Thus, these experiments can show that the minigene serves to both: 1.) generate a CTL response and 2.) that the induced CTLs recognized cells expressing the encoded epitopes.

25 For example, to create a DNA sequence encoding the selected epitopes (minigene) for expression in human cells, the amino acid sequences of the epitopes may be reverse translated. A human codon usage table can be used to guide the codon choice for each amino acid. These epitope-encoding DNA sequences may be directly adjoined, so that when translated, a continuous polypeptide sequence is created. To optimize expression and/or immunogenicity, additional elements can be incorporated into the  
30 minigene design. Examples of amino acid sequences that can be reverse translated and included in the minigene sequence include: HLA class I epitopes, HLA class II epitopes, a ubiquitination signal sequence, and/or an endoplasmic reticulum targeting signal. In addition, HLA presentation of CTL and HTL epitopes may be improved by including synthetic (e.g. poly-alanine) or naturally-occurring flanking sequences adjacent to the CTL or HTL epitopes; these larger peptides comprising the epitope(s) are within the scope  
35 of the invention.

The minigene sequence may be converted to DNA by assembling oligonucleotides that  
40 encode the plus and minus strands of the minigene. Overlapping oligonucleotides (30-100 bases long) may be synthesized, phosphorylated, purified and annealed under appropriate conditions using well known techniques. The ends of the oligonucleotides can be joined, for example, using T4 DNA ligase. This synthetic minigene, encoding the epitope polypeptide, can then be cloned into a desired expression vector.

09/561,571



Standard regulatory sequences well known to those of skill in the art are preferably included in the vector to ensure expression in the target cells. Several vector elements are desirable: a promoter with a down-stream cloning site for minigene insertion; a polyadenylation signal for efficient transcription termination; an *E. coli* origin of replication; and an *E. coli* selectable marker (e.g. ampicillin or kanamycin resistance). Numerous promoters can be used for this purpose, e.g., the human cytomegalovirus (hCMV) promoter. See, e.g., U.S. Patent Nos. 5,580,859 and 5,589,466 for other suitable promoter sequences.

Additional vector modifications may be desired to optimize minigene expression and immunogenicity. In some cases, introns are required for efficient gene expression, and one or more synthetic or naturally-occurring introns could be incorporated into the transcribed region of the minigene. The inclusion of mRNA stabilization sequences and sequences for replication in mammalian cells may also be considered for increasing minigene expression.

Once an expression vector is selected, the minigene is cloned into the polylinker region downstream of the promoter. This plasmid is transformed into an appropriate *E. coli* strain, and DNA is prepared using standard techniques. The orientation and DNA sequence of the minigene, as well as all other elements included in the vector, are confirmed using restriction mapping and DNA sequence analysis. Bacterial cells harboring the correct plasmid can be stored as a master cell bank and a working cell bank.

In addition, immunostimulatory sequences (ISSs or CpGs) appear to play a role in the immunogenicity of DNA vaccines. These sequences may be included in the vector, outside the minigene coding sequence, if desired to enhance immunogenicity.

In some embodiments, a bi-cistronic expression vector which allows production of both the minigene-encoded epitopes and a second protein (included to enhance or decrease immunogenicity) can be used. Examples of proteins or polypeptides that could beneficially enhance the immune response if co-expressed include cytokines (e.g., IL-2, IL-12, GM-CSF), cytokine-inducing molecules (e.g., LeIF), costimulatory molecules, or for HTL responses, pan-DR binding proteins (PADRE™, Epimmune, San Diego, CA). Helper (HTL) epitopes can be joined to intracellular targeting signals and expressed separately from expressed CTL epitopes; this allows direction of the HTL epitopes to a cell compartment different than that of the CTL epitopes. If required, this could facilitate more efficient entry of HTL epitopes into the HLA class II pathway, thereby improving HTL induction. In contrast to HTL or CTL induction, specifically decreasing the immune response by co-expression of immunosuppressive molecules (e.g. TGF- $\beta$ ) may be beneficial in certain diseases.

Therapeutic quantities of plasmid DNA can be produced for example, by fermentation in *E. coli*, followed by purification. Aliquots from the working cell bank are used to inoculate growth medium, and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins supplied by QIAGEN, Inc. (Valencia, California). If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). This approach, known as "naked DNA," is currently being used for intramuscular (IM) administration in clinical

5 trials. To maximize the immunotherapeutic effects of minigene DNA vaccines, an alternative method for formulating purified plasmid DNA may be desirable. A variety of methods have been described, and new techniques may become available. Cationic lipids, glycolipids, and fusogenic liposomes can also be used in the formulation (see, e.g., as described by WO 93/24640; Mannino & Gould-Fogerite, *BioTechniques* 6(7): 682 (1988); U.S. Pat No. 5,279,833; WO 91/06309; and Felgner, *et al.*, *Proc. Nat'l Acad. Sci. USA* 84:7413 (1987). In addition, peptides and compounds referred to collectively as protective, interactive, non-condensing compounds (PINC) could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types.

10 Target cell sensitization can be used as a functional assay for expression and HLA class I presentation of minigene-encoded CTL epitopes. For example, the plasmid DNA is introduced into a mammalian cell line that is suitable as a target for standard CTL chromium release assays. The transfection method used will be dependent on the final formulation. Electroporation can be used for "naked" DNA, whereas cationic lipids allow direct *in vitro* transfection. A plasmid expressing green fluorescent protein (GFP) can be co-transfected to allow enrichment of transfected cells using fluorescence activated cell sorting (FACS). These cells are then chromium-51 ( $^{51}\text{Cr}$ ) labeled and used as target cells for epitope-specific CTL lines; cytolysis, detected by  $^{51}\text{Cr}$  release, indicates both production of, and HLA presentation of, minigene-encoded CTL epitopes. Expression of HTL epitopes may be evaluated in an analogous manner using assays to assess HTL activity.

20 *In vivo* immunogenicity is a second approach for functional testing of minigene DNA formulations. Transgenic mice expressing appropriate human HLA proteins are immunized with the DNA product. The dose and route of administration are formulation dependent (e.g., IM for DNA in PBS, intraperitoneal (i.p.) for lipid-complexed DNA). Twenty-one days after immunization, splenocytes are harvested and restimulated for one week in the presence of peptides encoding each epitope being tested. Thereafter, for CTL effector cells, assays are conducted for cytolysis of peptide-loaded,  $^{51}\text{Cr}$ -labeled target cells using standard techniques. Lysis of target cells that were sensitized by HLA loaded with peptide epitopes, corresponding to minigene-encoded epitopes, demonstrates DNA vaccine function for *in vivo* induction of CTLs. Immunogenicity of HTL epitopes is evaluated in transgenic mice in an analogous manner.

30 Alternatively, the nucleic acids can be administered using ballistic delivery as described, for instance, in U.S. Patent No. 5,204,253. Using this technique, particles comprised solely of DNA are administered. In a further alternative embodiment, DNA can be adhered to particles, such as gold particles.

Minigenes can also be delivered using other bacterial or viral delivery systems well known in the art, e.g., an expression construct encoding epitopes of the invention can be incorporated into a viral vector such as vaccinia.

### 35 III.K.2. Combinations of CTL Peptides with Helper Peptides

Vaccine compositions comprising CTL peptides of the invention can be modified to provide desired attributes, such as improved serum half life, broadened population coverage or enhanced immunogenicity.

For instance, the ability of a peptide to induce CTL activity can be enhanced by linking the peptide to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. The use of T helper epitopes in conjunction with CTL epitopes to enhance immunogenicity is illustrated, for example, in the co-pending applications U.S.S.N. 08/820,360, U.S.S.N. 08/197,484, and U.S.S.N. 08/464,234.

Although a CTL peptide can be directly linked to a T helper peptide, often CTL epitope/HTL epitope conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homooligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues and sometimes 10 or more residues. The CTL peptide epitope can be linked to the T helper peptide epitope either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated.

In certain embodiments, the T helper peptide is one that is recognized by T helper cells present in the majority of the population. This can be accomplished by selecting peptides that bind to many, most, or all of the HLA class II molecules. These are known as "loosely HLA-restricted" or "promiscuous" T helper sequences. Examples of amino acid sequences that are promiscuous include sequences from antigens such as tetanus toxoid at positions 830-843 (QYIKANSKFIGITE; SEQ ID NO: 51484), *Plasmodium falciparum* circumsporozoite (CS) protein at positions 378-398 (DIEKKIAKMEKASSVFNVNS; SEQ ID NO: 51485), and *Streptococcus* 18kD protein at positions 116 (GAVDSILGGVATYGAA; SEQ ID NO: 51486). Other examples include peptides bearing a DR 1-4-7 supermotif, or either of the DR3 motifs.

Alternatively, it is possible to prepare synthetic peptides capable of stimulating T helper lymphocytes, in a loosely HLA-restricted fashion, using amino acid sequences not found in nature (see, e.g., PCT publication WO 95/07707). These synthetic compounds called Pan-DR-binding epitopes (e.g., PADRE™, Epimmune, Inc., San Diego, CA) are designed to most preferably bind most HLA-DR (human HLA class II) molecules. For instance, a pan-DR-binding epitope peptide having the formula: aKXVAAWTLKAAa, where "X" is either cyclohexylalanine, phenylalanine, or tyrosine, and a is either D-alanine or L-alanine, has been found to bind to most HLA-DR alleles, and to stimulate the response of T helper lymphocytes from most individuals, regardless of their HLA type. An alternative of a pan-DR binding epitope comprises all "L" natural amino acids and can be provided in the form of nucleic acids that encode the epitope.

HTL peptide epitopes can also be modified to alter their biological properties. For example, they can be modified to include D-amino acids to increase their resistance to proteases and thus extend their serum half life, or they can be conjugated to other molecules such as lipids, proteins, carbohydrates, and the like to increase their biological activity. For example, a T helper peptide can be conjugated to one or more palmitic acid chains at either the amino or carboxyl termini.

### III.K.3. Combinations of CTL Peptides with T Cell Priming Agents

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes cytotoxic T lymphocytes. Lipids have been identified as agents capable of priming CTL *in vivo* against viral antigens. For example, palmitic acid residues can be attached to the  $\epsilon$ - and  $\alpha$ - amino groups of a lysine residue and then linked, *e.g.*, via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be administered either directly in a micelle or particle, incorporated into a liposome, or emulsified in an adjuvant, *e.g.*, incomplete Freund's adjuvant. In a preferred embodiment, a particularly effective immunogenic composition comprises palmitic acid attached to  $\epsilon$ - and  $\alpha$ - amino groups of Lys, which is attached via linkage, *e.g.*, Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, *E. coli* lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine ( $P_3$ CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide (*see, e.g.*, Deres, *et al.*, *Nature* 342:561, 1989). Peptides of the invention can be coupled to  $P_3$ CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Moreover, because the induction of neutralizing antibodies can also be primed with  $P_3$ CSS-conjugated epitopes, two such compositions can be combined to more effectively elicit both humoral and cell-mediated responses.

CTL and/or HTL peptides can also be modified by the addition of amino acids to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide, particularly class I peptides. However, it is to be noted that modification at the carboxyl terminus of a CTL epitope may, in some cases, alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH<sub>2</sub> acylation, *e.g.*, by alkanoyl (C1-C20) or thioglycolyl acetylation, terminal-carboxyl amidation, *e.g.*, ammonia, methylamine, *etc.* In some instances these modifications may provide sites for linking to a support or other molecule.

### IV.J.4. Vaccine Compositions Comprising DC Pulsed with CTL and/or HTL Peptides

An embodiment of a vaccine composition in accordance with the invention comprises *ex vivo* administration of a cocktail of epitope-bearing peptides to PBMC, or isolated DC therefrom, from the patient's blood. A pharmaceutical to facilitate harvesting of DC can be used, such as Progenipoinetin (Monsanto, St. Louis, MO) or GM-CSF/IL-4. After pulsing the DC with peptides and prior to reinfusion into patients, the DC are washed to remove unbound peptides. In this embodiment, a vaccine comprises peptide-pulsed DCs which present the pulsed peptide epitopes complexed with HLA molecules on their surfaces.

The DC can be pulsed *ex vivo* with a cocktail of peptides, some of which stimulate CTL responses to one or more HPV antigens of interest. Optionally, a helper T cell (HTL) peptide such as a PADRE family molecule, can be included to facilitate the CTL response. Thus, a vaccine in accordance

with the invention, preferably comprising epitopes from multiple HPV antigens, is used to treat HPV infection or cancer resulting from HPV infection.

### III.L. Administration of Vaccines for Therapeutic or Prophylactic Purposes

5 The peptides of the present invention and pharmaceutical and vaccine compositions of the invention are typically used to treat and/or prevent cancer associated with HPV infection. Vaccine compositions containing the peptides of the invention are administered to a patient infected with HPV or to an individual susceptible to, or otherwise at risk for, HPV infection to elicit an immune response against HPV antigens and thus enhance the patient's own immune response capabilities.

10 As noted above, peptides comprising CTL and/or HTL epitopes of the invention induce immune responses when presented by HLA molecules and contacted with a CTL or HTL specific for an epitope comprised by the peptide. The peptides (or DNA encoding them) can be administered individually or as fusions of one or more peptide sequences. The manner in which the peptide is contacted with the CTL or HTL is not critical to the invention. For instance, the peptide can be contacted with the CTL or HTL  
15 either *in vivo* or *in vitro*. If the contacting occurs *in vivo*, the peptide itself can be administered to the patient, or other vehicles, *e.g.*, DNA vectors encoding one or more peptides, viral vectors encoding the peptide(s), liposomes and the like, can be used, as described herein.

When the peptide is contacted *in vitro*, the vaccinating agent can comprise a population of cells, *e.g.*, peptide-pulsed dendritic cells, or HPV-specific CTLs, which have been induced by pulsing  
20 antigen-presenting cells *in vitro* with the peptide or by transfecting antigen-presenting cells with a minigene of the invention. Such a cell population is subsequently administered to a patient in a therapeutically effective dose.

In therapeutic applications, peptide and/or nucleic acid compositions are administered to a patient in an amount sufficient to elicit an effective CTL and/or HTL response to the virus antigen and to  
25 cure or at least partially arrest or slow symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular composition administered, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

30 For pharmaceutical compositions, the immunogenic peptides of the invention, or DNA encoding them, are generally administered to an individual already infected with HPV. The peptides or DNA encoding them can be administered individually or as fusions of one or more peptide sequences. HPV-infected patients, with or without neoplasia, can be treated with the immunogenic peptides separately or in conjunction with other treatments, such as surgery, as appropriate.

35 For therapeutic use, administration should generally begin at the first diagnosis of HPV infection or HPV-associated cancer. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. The embodiment of the vaccine composition (*i.e.*, including, but not limited to embodiments such as peptide cocktails, polyepitopic polypeptides, minigenes, or TAA-specific CTLs or pulsed dendritic cells) delivered to the patient may vary according to the stage of  
40 the disease or the patient's health status. For example, in a patient with a tumor that expresses HPV

antigens, a vaccine comprising HPV-specific CTL may be more efficacious in killing tumor cells in patient with advanced disease than alternative embodiments.

Where susceptible individuals are identified prior to or during infection, the composition can be targeted to them, thus minimizing the need for administration to a larger population. Susceptible populations include those individuals who are sexually active.

The peptide or other compositions used for the treatment or prophylaxis of HPV infection can be used, *e.g.*, in persons who have not manifested symptoms, *e.g.*, genital warts or neoplastic growth. In this context, it is generally important to provide an amount of the peptide epitope delivered by a mode of administration sufficient to effectively stimulate a cytotoxic T cell response; compositions which stimulate helper T cell responses can also be given in accordance with this embodiment of the invention.

The dosage for an initial therapeutic immunization generally occurs in a unit dosage range where the lower value is about 1, 5, 50, 500, or 1,000  $\mu\text{g}$  and the higher value is about 10,000; 20,000; 30,000; or 50,000  $\mu\text{g}$ . Dosage values for a human typically range from about 500  $\mu\text{g}$  to about 50,000  $\mu\text{g}$  per 70 kilogram patient. Boosting dosages of between about 1.0  $\mu\text{g}$  to about 50,000  $\mu\text{g}$  of peptide pursuant to a boosting regimen over weeks to months may be administered depending upon the patient's response and condition as determined by measuring the specific activity of CTL and HTL obtained from the patient's blood. Administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection, or neoplasia, has been eliminated or reduced and for a period thereafter. The dosages, routes of administration, and dose schedules are adjusted in accordance with methodologies known in the art.

In certain embodiments, the peptides and compositions of the present invention are employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, as a result of the minimal amounts of extraneous substances and the relative nontoxic nature of the peptides in preferred compositions of the invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions relative to these stated dosage amounts.

The vaccine compositions of the invention can also be used purely as prophylactic agents. Generally the dosage for an initial prophylactic immunization generally occurs in a unit dosage range where the lower value is about 1, 5, 50, 500, or 1000  $\mu\text{g}$  and the higher value is about 10,000; 20,000; 30,000; or 50,000  $\mu\text{g}$ . Dosage values for a human typically range from about 500  $\mu\text{g}$  to about 50,000  $\mu\text{g}$  per 70 kilogram patient. This is followed by boosting dosages of between about 1.0  $\mu\text{g}$  to about 50,000  $\mu\text{g}$  of peptide administered at defined intervals from about four weeks to six months after the initial administration of vaccine. The immunogenicity of the vaccine can be assessed by measuring the specific activity of CTL and HTL obtained from a sample of the patient's blood.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral, intrathecal, or local (*e.g.* as a cream or topical ointment) administration. Preferably, the pharmaceutical compositions are administered parentally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional,

well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH-adjusting and buffering agents, tonicity  
5 adjusting agents, wetting agents, preservatives, and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.*

The concentration of peptides of the invention in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the  
10 particular mode of administration selected.

A human unit dose form of the peptide composition is typically included in a pharmaceutical composition that comprises a human unit dose of an acceptable carrier, preferably an aqueous carrier, and is administered in a volume of fluid that is known by those of skill in the art to be used for administration of such compositions to humans (*see, e.g., Remington's Pharmaceutical Sciences*, 17<sup>th</sup>  
15 Edition, A. Gennaro, Editor, Mack Publishing Co., Easton, Pennsylvania, 1985).

The peptides of the invention, and/or nucleic acids encoding the peptides, can also be administered via liposomes, which may also serve to target the peptides to a particular tissue, such as lymphoid tissue, or to target selectively to infected cells, as well as to increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals,  
20 phospholipid dispersions, lamellar layers and the like. In these preparations, the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the  
25 peptide compositions. Liposomes for use in accordance with the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), and U.S. Patent  
30 Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

For targeting cells of the immune system, a ligand to be incorporated into the liposome can include, *e.g.*, antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, *etc.* in a dose which varies according to, *inter alia*, the manner of administration, the  
35 peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally

employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

### III.M. HLA EXPRESSION: IMPLICATIONS FOR T CELL-BASED IMMUNOTHERAPY

#### Disease progression in cancer and infectious disease

It is well recognized that a dynamic interaction between exists between host and disease, both in the cancer and infectious disease settings. In the infectious disease setting, it is well established that pathogens evolve during disease. The strains that predominate early in HIV infection are different from the ones that are associated with AIDS and later disease stages (NS versus S strains). It has long been hypothesized that pathogen forms that are effective in establishing infection may differ from the ones most effective in terms of replication and chronicity.

Similarly, it is widely recognized that the pathological process by which an individual succumbs to a neoplastic disease is complex. During the course of disease, many changes occur in cancer cells. The tumor accumulates alterations which are in part related to dysfunctional regulation of growth and differentiation, but also related to maximizing its growth potential, escape from drug treatment and/or the body's immunosurveillance. Neoplastic disease results in the accumulation of several different biochemical alterations of cancer cells, as a function of disease progression. It also results in significant levels of intra- and inter- cancer heterogeneity, particularly in the late, metastatic stage.

Familiar examples of cellular alterations affecting treatment outcomes include the outgrowth of radiation or chemotherapy resistant tumors during the course of therapy. These examples parallel the emergence of drug resistant viral strains as a result of aggressive chemotherapy, *e.g.*, of chronic HBV and HIV infection, and the current resurgence of drug resistant organisms that cause Tuberculosis and Malaria. It appears that significant heterogeneity of responses is also associated with other approaches to cancer therapy, including anti-angiogenesis drugs, passive antibody immunotherapy, and active T cell-based immunotherapy. Thus, in view of such phenomena, epitopes from multiple disease-related antigens can be used in vaccines and therapeutics thereby counteracting the ability of diseased cells to mutate and escape treatment.

#### The interplay between disease and the immune system

One of the main factors contributing to the dynamic interplay between host and disease is the immune response mounted against the pathogen, infected cell, or malignant cell. In many conditions



such immune responses control the disease. Several animal model systems and prospective studies of natural infection in humans suggest that immune responses against a pathogen can control the pathogen, prevent progression to severe disease and/or eliminate the pathogen. A common theme is the requirement for a multispecific T cell response, and that narrowly focused responses appear to be less effective. These observations guide skilled artisan as to embodiments of methods and compositions of the present invention that provide for a broad immune response.

In the cancer setting there are several findings that indicate that immune responses can impact neoplastic growth:

First, the demonstration in many different animal models, that anti-tumor T cells, restricted by MHC class I, can prevent or treat tumors.

Second, encouraging results have come from immunotherapy trials.

Third, observations made in the course of natural disease correlated the type and composition of T cell infiltrate within tumors with positive clinical outcomes (Coulie PG, *et al.* Antitumor immunity at work in a melanoma patient In *Advances in Cancer Research*, 213-242, 1999).

Finally, tumors commonly have the ability to mutate, thereby changing their immunological recognition. For example, the presence of monospecific CTL was also correlated with control of tumor growth, until antigen loss emerged (Riker A, *et al.*, Immune selection after antigen-specific immunotherapy of melanoma *Surgery*, Aug: 126(2):112-20, 1999; Marchand M, *et al.*, Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1 *Int. J. Cancer* 80(2):219-30, Jan. 18, 1999). Similarly, loss of beta 2 microglobulin was detected in 5/13 lines established from melanoma patients after receiving immunotherapy at the NCI (Restifo NP, *et al.*, Loss of functional Beta2 - microglobulin in metastatic melanomas from five patients receiving immunotherapy *Journal of the National Cancer Institute*, Vol. 88 (2), 100-108, Jan. 1996). It has long been recognized that HLA class I is frequently altered in various tumor types. This has led to a hypothesis that this phenomenon might reflect immune pressure exerted on the tumor by means of class I restricted CTL. The extent and degree of alteration in HLA class I expression appears to be reflective of past immune pressures, and may also have prognostic value (van Duinen SG, *et al.*, Level of HLA antigens in locoregional metastases and clinical course of the disease in patients with melanoma *Cancer Research* 48, 1019-1025, Feb. 1988; Möller P, *et al.*, Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma *Cancer Research* 51, 729-736, Jan. 1991). Taken together, these observations provide a rationale for immunotherapy of cancer and infectious disease, and suggest that effective strategies need to account for the complex series of pathological changes associated with disease.

#### The three main types of alterations in HLA expression in tumors and their functional significance

The level and pattern of expression of HLA class I antigens in tumors has been studied in many different tumor types and alterations have been reported in all types of tumors studied. The molecular mechanisms underlining HLA class I alterations have been demonstrated to be quite heterogeneous. They include alterations in the TAP/processing pathways, mutations of  $\beta 2$ -microglobulin and specific HLA heavy chains, alterations in the regulatory elements controlling over class I expression and loss of entire

chromosome sections. There are several reviews on this topic, *see, e.g.*, : Garrido F, *et al.*, Natural history of HLA expression during tumour development *Immunol Today* 14(10):491-499, 1993; Kaklamani L, *et al.*, Loss of HLA class-I alleles, heavy chains and  $\beta$ 2-microglobulin in colorectal cancer *Int. J. Cancer*, 51(3):379-85, May 28, 1992. There are three main types of HLA Class I alteration (complete loss, allele-specific loss and decreased expression). The functional significance of each alteration is discussed separately:

#### *Complete loss of HLA expression*

Complete loss of HLA expression can result from a variety of different molecular mechanisms, reviewed in (Algarra I, *et al.*, The HLA crossroad in tumor immunology *Human Immunology* 61, 65-73, 2000; Browning M, *et al.*, Mechanisms of loss of HLA class I expression on colorectal tumor cells *Tissue Antigens* 47:364-371, 1996; Ferrone S, *et al.*, Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance *Immunology Today*, 16(10): 487-494, 1995; Garrido F, *et al.*, Natural history of HLA expression during tumour development *Immunology Today* 14(10):491-499, 1993; Tait, BD, HLA Class I expression on human cancer cells: Implications for effective immunotherapy *Hum Immunol* 61, 158-165, 2000). In functional terms, this type of alteration has several important implications.

While the complete absence of class I expression will eliminate CTL recognition of those tumor cells, the loss of HLA class I will also render the tumor cells extraordinary sensitive to lysis from NK cells (Ohnmacht, GA, *et al.*, Heterogeneity in expression of human leukocyte antigens and melanoma-associated antigens in advanced melanoma *J Cellular Phys* 182:332-338, 2000; Liunggren HG, *et al.*, Host resistance directed selectively against H-2 deficient lymphoma variants: Analysis of the mechanism *J. Exp. Med.*, Dec 1;162(6):1745-59, 1985; Maio M, *et al.*, Reduction in susceptibility to natural killer cell-mediated lysis of human FO-1 melanoma cells after induction of HLA class I antigen expression by transfection with B2m gene *J. Clin. Invest.* 88(1):282-9, July 1991; Schrier PI, *et al.*, Relationship between myc oncogene activation and MHC class I expression *Adv. Cancer Res.*, 60:181-246, 1993).

The complementary interplay between loss of HLA expression and gain in NK sensitivity is exemplified by the classic studies of Coulic and coworkers (Coulic, PG, *et al.*, Antitumor immunity at work in a melanoma patient. In *Advances in Cancer Research*, 213-242, 1999) which described the evolution of a patient's immune response over the course of several years. Because of increased sensitivity to NK lysis, it is predicted that approaches leading to stimulation of innate immunity in general and NK activity in particular would be of special significance. An example of such approach is the induction of large amounts of dendritic cells (DC) by various hematopoietic growth factors, such as Flt3 ligand or ProGP. The rationale for this approach resides in the well known fact that dendritic cells produce large amounts of IL-12, one of the most potent stimulators for innate immunity and NK activity in particular. Alternatively, IL-12 is administered directly, or as nucleic acids that encode it. In this light, it is interesting to note that Flt3 ligand treatment results in transient tumor regression of a class I negative prostate murine cancer model (Ciavarrá RP, *et al.*, Flt3-Ligand induces transient tumor regression in an ectopic treatment model of major histocompatibility complex-negative prostate cancer *Cancer Res* 60:2081-84, 2000). In this context, specific anti-tumor vaccines in accordance with the invention synergize with these types of

hematopoietic growth factors to facilitate both CTL and NK cell responses, thereby appreciably impairing a cell's ability to mutate and thereby escape efficacious treatment. Thus, an embodiment of the present invention comprises a composition of the invention together with a method or composition that augments functional activity or numbers of NK cells. Such an embodiment can comprise a protocol that provides a composition of the invention sequentially with an NK-inducing modality, or contemporaneous with an NK-inducing modality.

Secondly, complete loss of HLA frequently occurs only in a fraction of the tumor cells, while the remainder of tumor cells continue to exhibit normal expression. In functional terms, the tumor would still be subject, in part, to direct attack from a CTL response; the portion of cells lacking HLA subject to an NK response. Even if only a CTL response were used, destruction of the HLA expressing fraction of the tumor has dramatic effects on survival times and quality of life.

It should also be noted that in the case of heterogeneous HLA expression, both normal HLA-expressing as well as defective cells are predicted to be susceptible to immune destruction based on "bystander effects." Such effects were demonstrated, e.g., in the studies of Rosendahl and colleagues that investigated in vivo mechanisms of action of antibody targeted superantigens (Rosendahl A, *et al.*, Perforin and IFN-gamma are involved in the antitumor effects of antibody-targeted superantigens *J. Immunol.* 160(11):5309-13, June 1, 1998). The bystander effect is understood to be mediated by cytokines elicited from, e.g., CTLs acting on an HLA-bearing target cell, whereby the cytokines are in the environment of other diseased cells that are concomitantly killed.

#### *Allele-specific loss*

One of the most common types of alterations in class I molecules is the selective loss of certain alleles in individuals heterozygous for HLA. Allele-specific alterations might reflect the tumor adaptation to immune pressure, exerted by an immunodominant response restricted by a single HLA restriction element. This type of alteration allows the tumor to retain class I expression and thus escape NK cell recognition, yet still be susceptible to a CTL-based vaccine in accordance with the invention which comprises epitopes corresponding to the remaining HLA type. Thus, a practical solution to overcome the potential hurdle of allele-specific loss relies on the induction of multispecific responses. Just as the inclusion of multiple disease-associated antigens in a vaccine of the invention guards against mutations that yield loss of a specific disease antigens, simultaneously targeting multiple HLA specificities and multiple disease-related antigens prevents disease escape by allele-specific losses.

#### *Decrease in expression (allele-specific or not)*

The sensitivity of effector CTL has long been demonstrated (Brower, RC, *et al.*, Minimal requirements for peptide mediated activation of CD8+ CTL *Mol. Immunol.*, 31:1285-93, 1994; Chriustnick, ET, *et al.* Low numbers of MHC class I-peptide complexes required to trigger a T cell response *Nature* 352:67-70, 1991; Sykulev, Y, *et al.*, Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response *Immunity*, 4(6):565-71, June 1996). Even a single peptide/MHC complex can result in tumor cells lysis and release of anti-tumor lymphokines. The biological significance of decreased HLA expression and possible tumor escape from immune recognition is not fully known. Nevertheless, it

has been demonstrated that CTL recognition of as few as one MHC/peptide complex is sufficient to lead to tumor cell lysis.

Further, it is commonly observed that expression of HLA can be upregulated by gamma IFN, commonly secreted by effector CTL. Additionally, HLA class I expression can be induced in vivo by both alpha and beta IFN (Halloran, *et al.* Local T cell responses induce widespread MHC expression. *J. Immunol* 148:3837, 1992; Pestka, S, *et al.*, Interferons and their actions *Annu. Rev. Biochem.* 56:727-77, 1987). Conversely, decreased levels of HLA class I expression also render cells more susceptible to NK lysis.

With regard to gamma IFN, Torres et al (Torres, MJ, *et al.*, Loss of an HLA haplotype in pancreas cancer tissue and its corresponding tumor derived cell line. *Tissue Antigens* 47:372-81, 1996) note that HLA expression is upregulated by gamma IFN in pancreatic cancer, unless a total loss of haplotype has occurred. Similarly, Rees and Mian note that allelic deletion and loss can be restored, at least partially, by cytokines such as IFN-gamma (Rees, R., *et al.* Selective MHC expression in tumours modulates adaptive and innate antitumour responses *Cancer Immunol Immunother* 48:374-81, 1999). It has also been noted that IFN-gamma treatment results in upregulation of class I molecules in the majority of the cases studied (Browning M, *et al.*, Mechanisms of loss of HLA class I expression on colorectal tumor cells. *Tissue Antigens* 47:364-71, 1996). Kaklamakis, et al. also suggested that adjuvant immunotherapy with IFN-gamma may be beneficial in the case of HLA class I negative tumors (Kaklamanis L, Loss of transporter in antigen processing 1 transport protein and major histocompatibility complex class I molecules in metastatic versus primary breast cancer. *Cancer Research* 55:5191-94, November 1995). It is important to underline that IFN-gamma production is induced and self-amplified by local inflammation/immunization (Halloran, *et al.* Local T cell responses induce widespread MHC expression *J. Immunol* 148:3837, 1992), resulting in large increases in MHC expressions even in sites distant from the inflammatory site.

Finally, studies have demonstrated that decreased HLA expression can render tumor cells more susceptible to NK lysis (Ohnmacht, GA, *et al.*, Heterogeneity in expression of human leukocyte antigens and melanoma-associated antigens in advanced melanoma *J Cellular Phys* 182:332-38, 2000; Liunggren HG, *et al.*, Host resistance directed selectively against H-2 deficient lymphoma variants: Analysis of the mechanism *J. Exp. Med.*, 162(6):1745-59, December 1, 1985; Maio M, *et al.*, Reduction in susceptibility to natural killer cell-mediated lysis of human FO-1 melanoma cells after induction of HLA class I antigen expression by transfection with  $\beta 2m$  gene *J. Clin. Invest.* 88(1):282-9, July 1991; Schrier PI, *et al.*, Relationship between myc oncogene activation and MHC class I expression *Adv. Cancer Res.*, 60:181-246, 1993). If decreases in HLA expression benefit a tumor because it facilitates CTL escape, but render the tumor susceptible to NK lysis, then a minimal level of HLA expression that allows for resistance to NK activity would be selected for (Garrido F, *et al.*, Implications for immunosurveillance of altered HLA class I phenotypes in human tumours *Immunol Today* 18(2):89-96, February 1997). Therefore, a therapeutic compositions or methods in accordance with the invention together with a treatment to upregulate HLA expression and/or treatment with high affinity T-cells renders the tumor sensitive to CTL destruction.

Frequency of alterations in HLA expression

The frequency of alterations in class I expression is the subject of numerous studies (Algarra I, *et al.*, The HLA crossroad in tumor immunology *Human Immunology* 61, 65-73, 2000). Rees and Mian estimate allelic loss to occur overall in 3-20% of tumors, and allelic deletion to occur in 15-50% of tumors. It should be noted that each cell carries two separate sets of class I genes, each gene carrying one HLA-A and one HLA-B locus. Thus, fully heterozygous individuals carry two different HLA-A molecules and two different HLA-B molecules. Accordingly, the actual frequency of losses for any specific allele could be as little as one quarter of the overall frequency. They also note that, in general, a gradient of expression exists between normal cells, primary tumors and tumor metastasis. In a study from Natali and coworkers (Natali PG, *et al.*, Selective changes in expression of HLA class I polymorphic determinants in human solid tumors *PNAS USA* 86:6719-6723, September 1989), solid tumors were investigated for total HLA expression, using W6/32 antibody, and for allele-specific expression of the A2 antigen, as evaluated by use of the BB7.2 antibody. Tumor samples were derived from primary cancers or metastasis, for 13 different tumor types, and scored as negative if less than 20%, reduced if in the 30-80% range, and normal above 80%. All tumors, both primary and metastatic, were HLA positive with W6/32. In terms of A2 expression, a reduction was noted in 16.1 % of the cases, and A2 was scored as undetectable in 39.4 % of the cases. Garrido and coworkers (Garrido F, *et al.*, Natural history of HLA expression during tumour development *Immunol Today* 14(10):491-99, 1993) emphasize that HLA changes appear to occur at a particular step in the progression from benign to most aggressive. Jiminez *et al* (Jiminez P, *et al.*, Microsatellite instability analysis in tumors with different mechanisms for total loss of HLA expression. *Cancer Immunol Immunother* 48:684-90, 2000) have analyzed 118 different tumors (68 colorectal, 34 laryngeal and 16 melanomas). The frequencies reported for total loss of HLA expression were 11% for colon, 18% for melanoma and 13 % for larynx. Thus, HLA class I expression is altered in a significant fraction of the tumor types, possibly as a reflection of immune pressure, or simply a reflection of the accumulation of pathological changes and alterations in diseased cells.

Immunotherapy in the context of HLA loss

A majority of the tumors express HLA class I, with a general tendency for the more severe alterations to be found in later stage and less differentiated tumors. This pattern is encouraging in the context of immunotherapy, especially considering that: 1) the relatively low sensitivity of immunohistochemical techniques might underestimate HLA expression in tumors; 2) class I expression can be induced in tumor cells as a result of local inflammation and lymphokine release; and, 3) class I negative cells are sensitive to lysis by NK cells.

Accordingly, various embodiments of the present invention can be selected in view of the fact that there can be a degree of loss of HLA molecules, particularly in the context of neoplastic disease. For example, the treating physician can assay a patient's tumor to ascertain whether HLA is being expressed. If a percentage of tumor cells express no class I HLA, then embodiments of the present invention that comprise methods or compositions that elicit NK cell responses can be employed. As noted herein, such NK-inducing methods or composition can comprise a Flt3 ligand or ProGP which facilitate mobilization of dendritic cells, the rationale being that dendritic cells produce large amounts of IL-12. IL-

12 can also be administered directly in either amino acid or nucleic acid form. It should be noted that compositions in accordance with the invention can be administered concurrently with NK cell-inducing compositions, or these compositions can be administered sequentially.

5 In the context of allele-specific HLA loss, a tumor retains class I expression and may thus escape NK cell recognition, yet still be susceptible to a CTL-based vaccine in accordance with the invention which comprises epitopes corresponding to the remaining HLA type. The concept here is analogous to embodiments of the invention that include multiple disease antigens to guard against mutations that yield loss of a specific antigen. Thus, one can simultaneously target multiple HLA specificities and epitopes from multiple disease-related antigens to prevent tumor escape by allele-specific loss as well as disease-  
10 related antigen loss. In addition, embodiments of the present invention can be combined with alternative therapeutic compositions and methods. Such alternative compositions and methods comprise, without limitation, radiation, cytotoxic pharmaceuticals, and/or compositions/methods that induce humoral antibody responses.

Moreover, it has been observed that expression of HLA can be upregulated by gamma  
15 IFN, which is commonly secreted by effector CTL, and that HLA class I expression can be induced in vivo by both alpha and beta IFN. Thus, embodiments of the invention can also comprise alpha, beta and/or gamma IFN to facilitate upregulation of HLA.

#### 20 **III.N. REPRIEVE PERIODS FROM THERAPIES THAT INDUCE SIDE EFFECTS: "Scheduled Treatment Interruptions or Drug Holidays"**

Recent evidence has shown that certain patients infected with a pathogen, whom are initially treated with a therapeutic regimen to reduce pathogen load, have been able to maintain decreased pathogen load when removed from the therapeutic regimen, i.e., during a "drug holiday" (Rosenberg, E., *et al.*, Immune control of HIV-1 after early treatment of acute infection *Nature* 407:523-26, Sept. 28, 2000)  
25 As appreciated by those skilled in the art, many therapeutic regimens for both pathogens and cancer have numerous, often severe, side effects. During the drug holiday, the patient's immune system is keeping the disease in check. Methods for using compositions of the invention are used in the context of drug holidays for cancer and pathogenic infection.

For treatment of an infection, where therapies are not particularly immunosuppressive,  
30 compositions of the invention are administered concurrently with the standard therapy. During this period, the patient's immune system is directed to induce responses against the epitopes comprised by the present inventive compositions. Upon removal from the treatment having side effects, the patient is primed to respond to the infectious pathogen should the pathogen load begin to increase. Composition of the invention can be provided during the drug holiday as well.

35 For patients with cancer, many therapies are immunosuppressive. Thus, upon achievement of a remission or identification that the patient is refractory to standard treatment, then upon removal from the immunosuppressive therapy, a composition in accordance with the invention is administered. Accordingly, as the patient's immune system reconstitutes, precious immune resources are simultaneously directed against the cancer. Composition of the invention can also be administered concurrently with an  
40 immunosuppressive regimen if desired.

### III.O. Kits

The peptide and nucleic acid compositions of this invention can be provided in kit form together with instructions for vaccine administration. Typically the kit would include desired peptide compositions in a container, preferably in unit dosage form and instructions for administration. An alternative kit would include a minigene construct with desired nucleic acids of the invention in a container, preferably in unit dosage form together with instructions for administration. Lymphokines such as IL-2 or IL-12 may also be included in the kit. Other kit components that may also be desirable include, for example, a sterile syringe, booster dosages, and other desired excipients.

### III.P. Overview

Epitopes in accordance with the present invention were successfully used to induce an immune response. Immune responses with these epitopes have been induced by administering the epitopes in various forms. The epitopes have been administered as peptides, as nucleic acids, and as viral vectors comprising nucleic acids that encode the epitope(s) of the invention. Upon administration of peptide-based epitope forms, immune responses have been induced by direct loading of an epitope onto an empty HLA molecule that is expressed on a cell, and via internalization of the epitope and processing via the HLA class I pathway; in either event, the HLA molecule expressing the epitope was then able to interact with and induce a CTL response. Peptides can be delivered directly or using such agents as liposomes. They can additionally be delivered using ballistic delivery, in which the peptides are typically in a crystalline form. When DNA is used to induce an immune response, it is administered either as naked DNA, generally in a dose range of approximately 1-5mg, or via the ballistic "gene gun" delivery, typically in a dose range of approximately 10-100 µg. The DNA can be delivered in a variety of conformations, *e.g.*, linear, circular *etc.* Various viral vectors have also successfully been used that comprise nucleic acids which encode epitopes in accordance with the invention.

Accordingly compositions in accordance with the invention exist in several forms. Embodiments of each of these composition forms in accordance with the invention have been successfully used to induce an immune response.

One composition in accordance with the invention comprises a plurality of peptides. This plurality or cocktail of peptides is generally admixed with one or more pharmaceutically acceptable excipients. The peptide cocktail can comprise multiple copies of the same peptide or can comprise a mixture of peptides. The peptides can be analogs of naturally occurring epitopes. The peptides can comprise artificial amino acids and/or chemical modifications such as addition of a surface active molecule, *e.g.*, lipidation; acetylation, glycosylation, biotinylation, phosphorylation *etc.* The peptides can be CTL or HTL epitopes. In a preferred embodiment the peptide cocktail comprises a plurality of different CTL epitopes and at least one HTL epitope. The HTL epitope can be naturally or non-naturally (*e.g.*, PADRE®, Epimmune Inc., San Diego, CA). The number of distinct epitopes in an embodiment of the invention is generally a whole unit integer from one through one hundred fifty (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70,

71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, or 150).

An additional embodiment of a composition in accordance with the invention comprises a polypeptide multi-epitope construct, *i.e.*, a polyepitopic peptide. Polyepitopic peptides in accordance with the invention are prepared by use of technologies well-known in the art. By use of these known technologies, epitopes in accordance with the invention are connected one to another. The polyepitopic peptides can be linear or non-linear, *e.g.*, multivalent. These polyepitopic constructs can comprise artificial amino acids, spacing or spacer amino acids, flanking amino acids, or chemical modifications between adjacent epitope units. The polyepitopic construct can be a heteropolymer or a homopolymer. The polyepitopic constructs generally comprise epitopes in a quantity of any whole unit integer between 2-150 (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, or 150). The polyepitopic construct can comprise CTL and/or HTL epitopes. One or more of the epitopes in the construct can be modified, *e.g.*, by addition of a surface active material, *e.g.* a lipid, or chemically modified, *e.g.*, acetylation, *etc.* Moreover, bonds in the multiepitopic construct can be other than peptide bonds, *e.g.*, covalent bonds, ester or ether bonds, disulfide bonds, hydrogen bonds, ionic bonds *etc.*

Alternatively, a composition in accordance with the invention comprises construct which comprises a series, sequence, stretch, *etc.*, of amino acids that have homology to (*i.e.*, corresponds to or is contiguous with) to a native sequence. This stretch of amino acids comprises at least one subsequence of amino acids that, if cleaved or isolated from the longer series of amino acids, functions as an HLA class I or HLA class II epitope in accordance with the invention. In this embodiment, the peptide sequence is modified, so as to become a construct as defined herein, by use of any number of techniques known or to be provided in the art. The polyepitopic constructs can contain homology to a native sequence in any whole unit integer increment from 70-100%, *e.g.*, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or, 100 percent.

A further embodiment of a composition in accordance with the invention is an antigen presenting cell that comprises one or more epitopes in accordance with the invention. The antigen presenting cell can be a "professional" antigen presenting cell, such as a dendritic cell. The antigen presenting cell can comprise the epitope of the invention by any means known or to be determined in the art. Such means include pulsing of dendritic cells with one or more individual epitopes or with one or more peptides that comprise multiple epitopes, by nucleic acid administration such as ballistic nucleic acid delivery or by other techniques in the art for administration of nucleic acids, including vector-based, *e.g.* viral vector, delivery of nucleic acids.

Further embodiments of compositions in accordance with the invention comprise nucleic acids that encode one or more peptides of the invention, or nucleic acids which encode a polyepitopic peptide in accordance with the invention. As appreciated by one of ordinary skill in the art, various nucleic acids compositions will encode the same peptide due to the redundancy of the genetic code. Each of these nucleic acid compositions falls within the scope of the present invention. This embodiment of the invention



comprises DNA or RNA, and in certain embodiments a combination of DNA and RNA. It is to be appreciated that any composition comprising nucleic acids that will encode a peptide in accordance with the invention or any other peptide based composition in accordance with the invention, falls within the scope of this invention.

5 It is to be appreciated that peptide-based forms of the invention (as well as the nucleic acids that encode them) can comprise analogs of epitopes of the invention generated using principles already known, or to be known, in the art. Principles related to analoging are now known in the art, and are disclosed herein; moreover, analoging principles (heteroclitic analoging) are disclosed in co-pending application serial number U.S.S.N. 09/226,775 filed 6 January 1999. Generally the compositions of the  
10 invention are isolated or purified.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of non-critical parameters that can be  
15 changed or modified to yield alternative embodiments in accordance with the invention.

#### IV. EXAMPLES

The following example of peptide binding to HLA molecules demonstrates quantification of binding affinities of HLA class I and class II peptides. Binding assays can be performed with peptides  
20 that are either motif-bearing or not motif-bearing.

##### Example 1. HLA Class I and Class II Binding Assays

The following example of peptide binding to HLA molecules demonstrates quantification of binding affinities of HLA class I and class II peptides. Binding assays can be performed with peptides  
25 that are either motif-bearing or not motif-bearing.

HLA class I and class II binding assays using purified HLA molecules were performed in accordance with disclosed protocols (e.g., PCT publications WO 94/20127 and WO 94/03205; Sidney *et al.*, *Current Protocols in Immunology* 18.3.1 (1998); Sidney, *et al.*, *J. Immunol.* 154:247 (1995); Sette, *et al.*, *Mol. Immunol.* 31:813 (1994)). Briefly, purified MHC molecules (5 to 500nM) were incubated with  
30 various unlabeled peptide inhibitors and 1-10nM <sup>125</sup>I-radiolabeled probe peptides as described. Following incubation, MHC-peptide complexes were separated from free peptide by gel filtration and the fraction of peptide bound was determined. Typically, in preliminary experiments, each MHC preparation was titrated in the presence of fixed amounts of radiolabeled peptides to determine the concentration of HLA molecules necessary to bind 10-20% of the total radioactivity. All subsequent inhibition and direct binding assays  
35 were performed using these HLA concentrations.

Since under these conditions  $[label] < [HLA]$  and  $IC_{50} \geq [HLA]$ , the measured  $IC_{50}$  values are reasonable approximations of the true  $K_D$  values. Peptide inhibitors are typically tested at concentrations ranging from 120  $\mu$ g/ml to 1.2 ng/ml, and are tested in two to four completely independent experiments. To allow comparison of the data obtained in different experiments, a relative binding figure is  
40 calculated for each peptide by dividing the  $IC_{50}$  of a positive control for inhibition by the  $IC_{50}$  for each

- tested peptide (typically unlabeled versions of the radiolabeled probe peptide). For database purposes, and inter-experiment comparisons, relative binding values are compiled. These values can subsequently be converted back into  $IC_{50}$  nM values by dividing the  $IC_{50}$  nM of the positive controls for inhibition by the relative binding of the peptide of interest. This method of data compilation has proven to be the most accurate and consistent for comparing peptides that have been tested on different days, or with different lots of purified MHC.

Binding assays as outlined above may be used to analyze supermotif and/or motif-bearing epitopes as, for example, described in Example 2.

#### 10 Example 2. Identification of HLA Supermotif- and Motif-Bearing CTL Candidate Epitopes

Vaccine compositions of the invention can include multiple epitopes that comprise multiple HLA supermotifs or motifs to achieve broad population coverage. This example illustrates the identification of supermotif- and motif-bearing epitopes for the inclusion in such a vaccine composition. Calculation of population coverage was performed using the strategy described below.

15

#### *Computer searches and algorithms for identification of supermotif and/or motif-bearing epitopes*

The searches performed to identify the motif-bearing peptide sequences in Examples 2 and 5 employed the protein sequence data from seven proteins (E1, E2, E5, E6, E7, L1 and L2) from HPV types 16, 18, 31, 33, 45, and 56.

Accession numbers for HPV types			
Protein	6a	6b	11
E1	Q84293 AAA74213	P03113 CAA25020 W1WL6	W1WL11 P04014 AAA46929
E2	Q84294 AAA74214	P03119 CAA25021 W2WL6	AAA46930 W2WL11 P04015
E4	Q84295 AAA74215	CAA25022 W4WL6	P04016 W4WL11 AAA46931
E5a	Q84296 AAA74216	P06460 CAA25023 W5WL6A	W5WL11 P04017 AAA46932
E5b	N.A.	P06461 CAA25024 W5WLB	W5WL1B P04018 AAA46933
E6	Q84291 AAA74211	P06462 CAA25018 W6WL6	W6WL11 P04019 AAA21703 AAA46927
E7	Q84929 AAA74212	P06464 CAA25019 W7WL6	AAA46928 AAA21704 W7WL11 P04020
L1	P03100 AAA74218	P03100 CAA25026 P1WL6	P04012 P1WL11 AAA4635
L2	Q84297	P03106 CAA25025	P2WL11 AAA46934

		P2WL6	P040I3
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	<u>Strain</u>	<u>Protein Antigen</u>	<u>Accession number</u>
	HPV16	E1	W1SLHS
	HPV16	E2	W2WLHS
5	HPV16	E5	W5WLHS
	HPV16	E6	W6WLHS
	HPV16	E7	W7WLHS
	HPV16	L1	AAD33259
	HPV16	L2	AAD33258
10	HPV18	E1	W1WL18
	HPV18	E2	WL18
	HPV18	E5	W5WL18
	HPV18	E6	W6WL18
	HPV18	E7	PO6788
15	HPV18	L1	CAA28671
	HPV18	L2	P2WL18
	HPV31	E1	W1WL31
	HPV31	E2	W2WL3
	HPV31	E5	W5WL31
20	HPV31	E6	W6WL31
	HPV31	E7	W7WL31
	HPV31	L1	P1WL31
	HPV31	L2	P2WL31
	HPV45	E1	S36563
25	HPV45	E2	S36564
	HPV45	E6	CAB44706
	HPV45	E7	CAB44707
	HPV45	L1	CAB44705
	HPV45	L2	S36565
30	HPV33	E1	W1WL33
	HPV33	E2	W2WL33
	HPV33	E5	W5WL33
	HPV33	E6	W6WL33
	HPV33	E7	W7WL33
35	HPV33	L1	P1WL33
	HPV33	L2	P2WL33
	HPV56	E2	S36581
	HPV56	E6	W6WL56
	HPV56	E7	S36580
40	HPV56	L1	S38563

HPV56

L2

S36582

Computer searches for epitopes bearing HLA Class I or Class II supermotifs or motifs were performed as follows. All translated HPV protein sequences were analyzed using a text string search software program, e.g., MotifSearch 1.4 (D. Brown, San Diego) to identify potential peptide sequences containing appropriate HLA binding motifs; alternative programs are readily produced in accordance with information in the art in view of the motif/supermotif disclosure herein. Furthermore, such calculations can be made mentally.

Identified A2-, A3-, and DR-supermotif sequences were scored using polynomial algorithms to predict their capacity to bind to specific HLA-Class I or Class II molecules. These polynomial algorithms take into account both extended and refined motifs (that is, to account for the impact of different amino acids at different positions), and are essentially based on the premise that the overall affinity (or  $\Delta G$ ) of peptide-HLA molecule interactions can be approximated as a linear polynomial function of the type:

$$"\Delta G" = a_{1i} \times a_{2i} \times a_{3i} \dots \times a_{ni}$$

where  $a_{ji}$  is a coefficient which represents the effect of the presence of a given amino acid ( $j$ ) at a given position ( $i$ ) along the sequence of a peptide of  $n$  amino acids. The crucial assumption of this method is that the effects at each position are essentially independent of each other (i.e., independent binding of individual side-chains). When residue  $j$  occurs at position  $i$  in the peptide, it is assumed to contribute a constant amount  $j_i$  to the free energy of binding of the peptide irrespective of the sequence of the rest of the peptide. This assumption is justified by studies from our laboratories that demonstrated that peptides are bound to MHC and recognized by T cells in essentially an extended conformation (data omitted herein).

The method of derivation of specific algorithm coefficients has been described in Gulukota *et al.*, *J. Mol. Biol.* 267:1258-126, 1997; (see also Sidney *et al.*, *Human Immunol.* 45:79-93, 1996; and Southwood *et al.*, *J. Immunol.* 160:3363-3373, 1998). Briefly, for all  $i$  positions, anchor and non-anchor alike, the geometric mean of the average relative binding (ARB) of all peptides carrying  $j$  is calculated relative to the remainder of the group, and used as the estimate of  $j_i$ . For Class II peptides, if multiple alignments are possible, only the highest scoring alignment is utilized, following an iterative procedure. To calculate an algorithm score of a given peptide in a test set, the ARB values corresponding to the sequence of the peptide are multiplied. If this product exceeds a chosen threshold, the peptide is predicted to bind. Appropriate thresholds are chosen as a function of the degree of stringency of prediction desired.

### Selection of HLA-A2 supertype cross-reactive peptides

Complete protein sequences from the seven HPV structural and regulatory proteins of the HPV strains listed above were aligned, then scanned, utilizing motif identification software, to identify 9- and 10-mer sequences containing the HLA-A2-supermotif main anchor specificity.

HLA-A2 supermotif-bearing sequences are shown in Table VIII. Typically, these sequences are then scored using the A2 algorithm and the peptides corresponding to the positive-scoring

sequences are synthesized and tested for their capacity to bind purified HLA-A\*0201 molecules *in vitro* (HLA-A\*0201 is considered a prototype A2 supertype molecule).

Examples of peptides that bind to HLA-A\*0201 with  $IC_{50}$  values  $\leq 500$  nM are shown in Table VIII. These peptides are then tested for the capacity to bind to additional A2-supertype molecules (A\*0202, A\*0203, A\*0206, and A\*6802). Peptides that bind to at least three of the five A2-supertype alleles tested are typically deemed A2-supertype cross-reactive binders. Preferred peptides bind at an affinity equal to or less than 500 nM to three or more HLA-A2 supertype molecules.

#### *Selection of HLA-A3 supermotif-bearing epitopes*

The HPV protein sequences scanned above were also examined for the presence of peptides with the HLA-A3-supermotif primary anchors (Table IX).

Peptides corresponding to the supermotif-bearing sequences are then synthesized and tested for binding to HLA-A\*0301 and HLA-A\*1101 molecules, the two most prevalent A3-supertype alleles. The peptides that are found to bind one of the two alleles with binding affinities of  $\leq 500$  nM, often  $\leq 200$  nM, are then tested for binding cross-reactivity to the other common A3-supertype alleles (A\*3101, A\*3301, and A\*6801) to identify those that can bind at least three of the five HLA-A3-supertype molecules tested.

#### *Selection of HLA-B7 supermotif bearing epitopes*

The same HPV target antigen protein sequences were also analyzed for the presence of 9- or 10-mer peptides with the HLA-B7-supermotif (Table XI).

Corresponding peptides are synthesized and tested for binding to HLA-B\*0702, the most common B7-supertype allele (*i.e.*, the prototype B7 supertype allele). Peptides binding B\*0702 with  $IC_{50}$  of  $\leq 500$  nM are identified using standard methods. These peptides are then tested for binding to other common B7-supertype molecules (B\*3501, B\*5101, B\*5301, and B\*5401). Peptides capable of binding to three or more of the five B7-supertype alleles tested are thereby identified.

#### *Selection of A1 and A24 motif-bearing epitopes*

To further increase population coverage, HLA-A1 and -A24 epitopes can, for example, also be incorporated into potential vaccine constructs. An analysis of the protein sequence data from the HPV target antigens utilized above can also be performed to identify HLA-A1- and A24-motif-containing sequences.

High affinity and/or cross-reactive binding epitopes that bear other motif and/or supermotifs are identified using analogous methodology.

#### Example 3. Confirmation of Immunogenicity

Cross-reactive candidate CTL A2-supermotif-bearing peptides that are identified as described in Example 2 were selected for *in vitro* immunogenicity testing. Testing was performed using the following methodology:

**Target Cell Lines for Cellular Screening:**

The .221A2.1 cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human B-lymphoblastoid cell line 721.221, is used as the peptide-loaded target to measure activity of HLA-A2.1-restricted CTL. This cell line is grown in RPMI-1640 medium supplemented with antibiotics, sodium pyruvate, nonessential amino acids and 10% (v/v) heat inactivated FCS. Cells that express an antigen of interest, or transfectants comprising the gene encoding the antigen of interest, can be used as target cells to test the ability of peptide-specific CTLs to recognize endogenous antigen.

**Primary CTL Induction Cultures:**

*Generation of Dendritic Cells (DC):* PBMCs are thawed in RPMI with 30 g/ml DNase, washed twice and resuspended in complete medium (RPMI-1640 plus 5% AB human serum, non-essential amino acids, sodium pyruvate, L-glutamine and penicillin/streptomycin). The monocytes are purified by plating  $10 \times 10^6$  PBMC/well in a 6-well plate. After 2 hours at 37°C, the non-adherent cells are removed by gently shaking the plates and aspirating the supernatants. The wells are washed a total of three times with 3 ml RPMI to remove most of the non-adherent and loosely adherent cells. Three ml of complete medium containing 50 ng/ml of GM-CSF and 1,000 U/ml of IL-4 are then added to each well. TNF is added to the DCs on day 6 at 75 ng/ml and the cells are used for CTL induction cultures on day 7.

*Induction of CTL with DC and Peptide:* CD8<sup>+</sup> T-cells are isolated by positive selection with Dynal immunomagnetic beads (Dynabeads® M-450) and the detach-bead® reagent. Typically about 200-250  $\times 10^6$  PBMC are processed to obtain  $24 \times 10^6$  CD8<sup>+</sup> T-cells (enough for a 48-well plate culture). Briefly, the PBMCs are thawed in RPMI with 30  $\mu$ g/ml DNase, washed once with PBS containing 1% human AB serum and resuspended in PBS/1% AB serum at a concentration of  $20 \times 10^6$  cells/ml. The magnetic beads are washed 3 times with PBS/AB serum, added to the cells (140  $\mu$ l beads/ $20 \times 10^6$  cells) and incubated for 1 hour at 4°C with continuous mixing. The beads and cells are washed 4x with PBS/AB serum to remove the nonadherent cells and resuspended at  $100 \times 10^6$  cells/ml (based on the original cell number) in PBS/AB serum containing 100  $\mu$ l/ml detach-bead® reagent and 30  $\mu$ g/ml DNase. The mixture is incubated for 1 hour at room temperature with continuous mixing. The beads are washed again with PBS/AB/DNase to collect the CD8<sup>+</sup> T-cells. The DC are collected and centrifuged at 1300 rpm for 5-7 minutes, washed once with PBS with 1% BSA, counted and pulsed with 40  $\mu$ g/ml of peptide at a cell concentration of  $1-2 \times 10^6$ /ml in the presence of 3  $\mu$ g/ml  $\beta_2$ -microglobulin for 4 hours at 20°C. The DC are then irradiated (4,200 rads), washed 1 time with medium and counted again.

*Setting up induction cultures:* 0.25 ml cytokine-generated DC ( $1 \times 10^5$  cells/ml) are co-cultured with 0.25 ml of CD8<sup>+</sup> T-cells ( $2 \times 10^6$  cell/ml) in each well of a 48-well plate in the presence of 10 ng/ml of IL-7. Recombinant human IL10 is added the next day at a final concentration of 10 ng/ml and rhuman IL2 is added 48 hours later at 10 IU/ml.

*Restimulation of the induction cultures with peptide-pulsed adherent cells:* Seven and fourteen days after the primary induction the cells are restimulated with peptide-pulsed adherent cells. The PBMCs are thawed and washed twice with RPMI and DNase. The cells are resuspended at  $5 \times 10^6$  cells/ml and irradiated at ~4200 rads. The PBMCs are plated at  $2 \times 10^6$  in 0.5 ml complete medium per well and incubated for 2 hours at 37°C. The plates are washed twice with RPMI by tapping the plate gently to

remove the nonadherent cells and the adherent cells pulsed with 10 µg/ml of peptide in the presence of 3 µg/ml  $\beta_2$  microglobulin in 0.25 ml RPMI/5% AB per well for 2 hours at 37°C. Peptide solution from each well is aspirated and the wells are washed once with RPMI. Most of the media is aspirated from the induction cultures (CD8+ cells) and brought to 0.5 ml with fresh media. The cells are then transferred to the wells containing the peptide-pulsed adherent cells. Twenty four hours later rhuman IL10 is added at a final concentration of 10 ng/ml and rhuman IL2 is added the next day and again 2-3 days later at 50 IU/ml (Tsai *et al.*, *Critical Reviews in Immunology* 18(1-2):65-75, 1998). Seven days later the cultures are assayed for CTL activity in a  $^{51}\text{Cr}$  release assay. In some experiments the cultures are assayed for peptide-specific recognition in the *in situ* IFN $\gamma$  ELISA at the time of the second restimulation followed by assay of endogenous recognition 7 days later. After expansion, activity is measured in both assays for a side by side comparison.

#### Measurement of CTL lytic activity by $^{51}\text{Cr}$ release.

Seven days after the second restimulation, cytotoxicity is determined in a standard (5 hr)  $^{51}\text{Cr}$  release assay by assaying individual wells at a single E:T. Peptide-pulsed targets are prepared by incubating the cells with 10 µg/ml peptide overnight at 37°C.

Adherent target cells are removed from culture flasks with trypsin-EDTA. Target cells are labelled with 200 µCi of  $^{51}\text{Cr}$  sodium chromate (Dupont, Wilmington, DE) for 1 hour at 37°C. Labelled target cells are resuspended at  $10^6$  per ml and diluted 1:10 with K562 cells at a concentration of  $3.3 \times 10^6$ /ml (an NK-sensitive erythroblastoma cell line used to reduce non-specific lysis). Target cells (100 µl) and 100 µl of effectors are plated in 96 well round-bottom plates and incubated for 5 hours at 37°C. At that time, 100 µl of supernatant are collected from each well and percent lysis is determined according to the formula: [(cpm of the test sample - cpm of the spontaneous  $^{51}\text{Cr}$  release sample)/(cpm of the maximal  $^{51}\text{Cr}$  release sample - cpm of the spontaneous  $^{51}\text{Cr}$  release sample)] x 100. Maximum and spontaneous release are determined by incubating the labelled targets with 1% Triton X-100 and media alone, respectively. A positive culture is defined as one in which the specific lysis (sample - background) is 10% or higher in the case of individual wells and is 15% or more at the 2 highest E:T ratios when expanded cultures are assayed.

#### *In situ* Measurement of Human $\gamma\text{IFN}$ Production as an Indicator of Peptide-specific and Endogenous Recognition

Immulon 2 plates are coated with mouse anti-human IFN $\gamma$  monoclonal antibody (4 µg/ml 0.1M NaHCO $_3$ , pH 8.2) overnight at 4°C. The plates are washed with Ca $^{2+}$ , Mg $^{2+}$ -free PBS/0.05% Tween 20 and blocked with PBS/10% FCS for 2 hours, after which the CTLs (100 µl/well) and targets (100 µl/well) are added to each well, leaving empty wells for the standards and blanks (which received media only). The target cells, either peptide-pulsed or endogenous targets, are used at a concentration of  $1 \times 10^6$  cells/ml. The plates are incubated for 48 hours at 37°C with 5% CO $_2$ .

Recombinant human IFN $\gamma$  is added to the standard wells starting at 400 pg or 1200 pg/100 µl/well and the plate incubated for 2 hours at 37°C. The plates are washed and 100 µl of biotinylated mouse anti-human IFN $\gamma$  monoclonal antibody (2 µg/ml in PBS/3% FCS/0.05% Tween 20) are added and incubated for 2 hours at room temperature. After washing again, 100 µl HRP-streptavidin

- (1:4000) are added and the plates incubated for 1 hour at room temperature. The plates are then washed 6x with wash buffer, 100µl/well developing solution (TMB 1:1) are added, and the plates allowed to develop for 5-15 minutes. The reaction is stopped with 50 µl/well 1M H<sub>3</sub>PO<sub>4</sub> and read at OD450. A culture is considered positive if it measured at least 50 pg of IFN $\gamma$ /well above background and is twice the background level of expression.

- CTL Expansion.** Those cultures that demonstrate specific lytic activity against peptide-pulsed targets and/or tumor targets are expanded over a two week period with anti-CD3. Briefly, 5x10<sup>4</sup> CD8<sup>+</sup> cells are added to a T25 flask containing the following: 1x10<sup>6</sup> irradiated (4,200 rad) PBMC (autologous or allogeneic) per ml, 2x10<sup>5</sup> irradiated (8,000 rad) EBV- transformed cells per ml, and OKT3 (anti-CD3) at 30ng per ml in RPMI-1640 containing 10% (v/v) human AB serum, non-essential amino acids, sodium pyruvate, 25µM 2-mercaptoethanol, L-glutamine and penicillin/streptomycin. Rhuman IL2 is added 24 hours later at a final concentration of 200IU/ml and every 3 days thereafter with fresh media at 50IU/ml. The cells are split if the cell concentration exceeded 1x10<sup>6</sup>/ml and the cultures are assayed between days 13 and 15 at E:T ratios of 30, 10, 3 and 1:1 in the <sup>51</sup>Cr release assay or at 1x10<sup>6</sup>/ml in the *in situ* IFN assay using the same targets as before the expansion.

- Cultures are expanded in the absence of anti-CD3<sup>+</sup> as follows. Those cultures that demonstrate specific lytic activity against peptide and endogenous targets are selected and 5x10<sup>4</sup> CD8<sup>+</sup> cells are added to a T25 flask containing the following: 1x10<sup>6</sup> autologous PBMC per ml which have been peptide-pulsed with 10µg/ml peptide for 2 hours at 37°C and irradiated (4,200 rad); 2x10<sup>5</sup> irradiated (8,000 rad) EBV-transformed cells per ml RPMI-1640 containing 10%(v/v) human AB serum, non-essential AA, sodium pyruvate, 25mM 2-ME, L-glutamine and gentamicin.

#### *Immunogenicity of A2 supermotif-bearing peptides*

- A2-supermotif cross-reactive binding peptides are tested in the cellular assay for the ability to induce peptide-specific CTL in normal individuals. In this analysis, a peptide is typically considered to be an epitope if it induces peptide-specific CTLs in at least 2 donors (unless otherwise noted) and preferably, also recognizes the endogenously expressed peptide.

- Immunogenicity is additionally confirmed using PBMCs isolated from HPV-infected patients. Briefly, PBMCs are isolated from patients, re-stimulated with peptide-pulsed monocytes and assayed for the ability to recognize peptide-pulsed target cells as well as transfected cells endogenously expressing the antigen.

#### *Evaluation of A\*03/A11 immunogenicity*

- HLA-A3 supermotif-bearing cross-reactive binding peptides are also evaluated for immunogenicity using methodology analogous for that used to evaluate the immunogenicity of the HLA-A2 supermotif peptides.



*Evaluation of B7 immunogenicity*

Immunogenicity screening of the B7-supertype cross-reactive binding peptides identified in Example 2 are evaluated in a manner analogous to the evaluation of A2-and A3-supermotif-bearing peptides.

5

Peptides bearing other supermotifs/motifs, *e.g.*, HLA-A1, HLA-A24 *etc.* are also evaluated using similar methodology

Example 4. Implementation of the Extended Supermotif to Improve the Binding Capacity of Native Epitopes by Creating Analogs

10

HLA motifs and supermotifs (comprising primary and/or secondary residues) are useful in the identification and preparation of highly cross-reactive native peptides, as demonstrated herein. Moreover, the definition of HLA motifs and supermotifs also allows one to engineer highly cross-reactive epitopes by identifying residues within a native peptide sequence which can be analoged, or "fixed" to confer upon the peptide certain characteristics, *e.g.* greater cross-reactivity within the group of HLA molecules that comprise a supertype, and/or greater binding affinity for some or all of those HLA molecules. Examples of analoging peptides to exhibit modulated binding affinity are set forth in this example.

15

*Analoging at Primary Anchor Residues*

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Peptide engineering strategies are implemented to further increase the cross-reactivity of the epitopes. For example, on the basis of the data disclosed, *e.g.*, in related and co-pending U.S.S.N 09/226,775, the main anchors of A2-supermotif-bearing peptides are altered, for example, to introduce a preferred L, I, V, or M at position 2, and I or V at the C-terminus.

25

To analyze the cross-reactivity of the analog peptides, each engineered analog is initially tested for binding to the prototype A2 supertype allele A\*0201, then, if A\*0201 binding capacity is maintained, for A2-supertype cross-reactivity.

Alternatively, a peptide is tested for binding to one or all supertype members and then analogued to modulate binding affinity to any one (or more) of the supertype members to add population coverage.

30

The selection of analogs for immunogenicity in a cellular screening analysis is typically further restricted by the capacity of the parent peptide to bind at least weakly, *i.e.*, bind at an IC<sub>50</sub> of 5000nM or less, to three or more A2 supertype alleles. The rationale for this requirement is that the WT peptides must be present endogenously in sufficient quantity to be biologically relevant. Analoged peptides have been shown to have increased immunogenicity and cross-reactivity by T cells specific for the parent epitope (*see, e.g.*, Parkhurst *et al.*, *J. Immunol.* 157:2539, 1996; and Pogue *et al.*, *Proc. Natl. Acad. Sci. USA* 92:8166, 1995).

35

In the cellular screening of these peptide analogs, it is important to demonstrate that analog-specific CTLs are also able to recognize the wild-type peptide and, when possible, target cells that endogenously express the epitope.

40

#### Analoging of HLA-A3 and B7-supermotif-bearing peptides

Analogs of HLA-A3 supermotif-bearing epitopes are generated using strategies similar to those employed in analoging HLA-A2 supermotif-bearing peptides. For example, peptides binding to 3/5 of the A3-supertype molecules are engineered at primary anchor residues to possess a preferred residue (V, S, M, or A) at position 2.

The analog peptides are then tested for the ability to bind A\*03 and A\*11 (prototype A3 supertype alleles). Those peptides that demonstrate  $\leq 500$  nM binding capacity are then tested for A3-supertype cross-reactivity.

Similarly to the A2- and A3- motif bearing peptides, peptides binding 3 or more B7-supertype alleles can be improved, where possible, to achieve increased cross-reactive binding. B7 supermotif-bearing peptides are, for example, engineered to possess a preferred residue (V, I, L, or F) at the C-terminal primary anchor position, as demonstrated by Sidney *et al.* (*J. Immunol.* 157:3480-3490, 1996).

Analoging at primary anchor residues of other motif and/or supermotif-bearing epitopes is performed in a like manner.

The analog peptides are then be tested for immunogenicity, typically in a cellular screening assay. Again, it is generally important to demonstrate that analog-specific CTLs are also able to recognize the wild-type peptide and, when possible, targets that endogenously express the epitope.

#### Analoging at Secondary Anchor Residues

Moreover, HLA supermotifs are of value in engineering highly cross-reactive peptides and/or peptides that bind HLA molecules with increased affinity by identifying particular residues at secondary anchor positions that are associated with such properties. For example, the binding capacity of a B7 supermotif-bearing peptide with an F residue at position 1 is analyzed. The peptide is then analoged to, for example, substitute L for F at position 1. The analoged peptide is evaluated for increased binding affinity/ and or increased cross-reactivity. Such a procedure identifies analoged peptides with modulated binding affinity.

Engineered analogs with sufficiently improved binding capacity or cross-reactivity can also be tested for immunogenicity in HLA-B7-transgenic mice, following for example, IFA immunization or lipopeptide immunization. Analogued peptides are additionally tested for the ability to stimulate a recall response using PBMC from HPV-infected patients.

#### Other analoguing strategies

Another form of peptide analoguing, unrelated to the anchor positions, involves the substitution of a cysteine with  $\alpha$ -amino butyric acid. Due to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally so as to reduce binding capacity. Substitution of  $\alpha$ -amino butyric acid for cysteine not only alleviates this problem, but has been shown to improve binding and crossbinding capabilities in some instances (*see, e.g.,* the review by Sette *et al.*, In: Persistent Viral Infections, Eds. R. Ahmed and I. Chen, John Wiley & Sons, England, 1999).

Thus, by the use of even single amino acid substitutions, the binding affinity and/or cross-reactivity of peptide ligands for HLA supertype molecules can be modulated.

Example 5. Identification of HPV-derived sequences with HLA-DR binding motifs

- 5 Peptide epitopes bearing an HLA class II supermotif or motif are identified as outlined below using methodology similar to that described in Examples 1-3.

*Selection of HLA-DR-supermotif-bearing epitopes.*

- 10 To identify HPV-derived, HLA class II HTL epitopes, the protein sequences from the same HPV antigens used for the identification of HLA Class I supermotif/motif sequences were analyzed for the presence of sequences bearing an HLA-DR-motif or supermotif. Specifically, 15-mer sequences were selected comprising a DR-supermotif, further comprising a 9-mer core, and three-residue N- and C-terminal flanking regions (15 amino acids total).

- 15 Protocols for predicting peptide binding to DR molecules have been developed (Southwood *et al.*, *J. Immunol.* 160:3363-3373, 1998). These protocols, specific for individual DR molecules, allow the scoring, and ranking, of 9-mer core regions. Each protocol not only scores peptide sequences for the presence of DR-supermotif primary anchors (i.e., at position 1 and position 6) within a 9-mer core, but additionally evaluates sequences for the presence of secondary anchors. Using allele specific selection tables (see, e.g., Southwood *et al.*, *ibid.*), it has been found that these protocols efficiently select  
20 peptide sequences with a high probability of binding a particular DR molecule. Additionally, it has been found that performing these protocols in tandem, specifically those for DR1, DR4w4, and DR7, can efficiently select DR cross-reactive peptides.

- The HPV-derived peptides identified above are tested for their binding capacity for various common HLA-DR molecules. All peptides are initially tested for binding to the DR molecules in  
25 the primary panel: DR1, DR4w4, and DR7. Peptides binding at least 2 of these 3 DR molecules are then tested for binding to DR2w2  $\beta$ 1, DR2w2  $\beta$ 2, DR6w19, and DR9 molecules in secondary assays. Finally, peptides binding at least 2 of the 4 secondary panel DR molecules, and thus cumulatively at least 4 of 7 different DR molecules, are screened for binding to DR4w15, DR5w11, and DR8w2 molecules in tertiary assays. Peptides binding at least 7 of the 10 DR molecules comprising the primary, secondary, and tertiary  
30 screening assays are considered cross-reactive DR binders. HPV-derived peptides found to bind common HLA-DR alleles are of particular interest.

*Selection of DR3 motif peptides*

- 35 Because HLA-DR3 is an allele that is prevalent in Caucasian, Black, and Hispanic populations, DR3 binding capacity is an important criterion in the selection of HTL epitopes. However, data generated previously indicated that DR3 only rarely cross-reacts with other DR alleles (Sidney *et al.*, *J. Immunol.* 149:2634-2640, 1992; Geluk *et al.*, *J. Immunol.* 152:5742-5748, 1994; Southwood *et al.*, *J. Immunol.* 160:3363-3373, 1998). This is not entirely surprising in that the DR3 peptide-binding motif appears to be distinct from the specificity of most other DR alleles. For maximum efficiency in developing  
40 vaccine candidates it would be desirable for DR3 motifs to be clustered in proximity with DR supermotif

regions. Thus, peptides shown to be candidates may also be assayed for their DR3 binding capacity. However, in view of the distinct binding specificity of the DR3 motif, peptides binding only to DR3 can also be considered as candidates for inclusion in a vaccine formulation.

To efficiently identify peptides that bind DR3, target HPV antigens are analyzed for sequences carrying one of the two DR3 specific binding motifs reported by Geluk *et al.* (*J. Immunol.* 152:5742-5748, 1994). The corresponding peptides are then synthesized and tested for the ability to bind DR3 with an affinity of 1  $\mu$ M or better, i.e., less than 1  $\mu$ M. Peptides are found that meet this binding criterion and qualify as HLA class II high affinity binders.

DR3 binding epitopes identified in this manner are included in vaccine compositions with DR supermotif-bearing peptide epitopes.

Similarly to the case of HLA class I motif-bearing peptides, the class II motif-bearing peptides are analoged to improve affinity or cross-reactivity. For example, aspartic acid at position 4 of the 9-mer core sequence is an optimal residue for DR3 binding, and substitution for that residue often improves DR 3 binding.

#### Example 6. Immunogenicity of HPV-derived HTL epitopes

This example determines immunogenic DR supermotif- and DR3 motif-bearing epitopes among those identified using the methodology in Example 5.

Immunogenicity of HTL epitopes are evaluated in a manner analogous to the determination of immunogenicity of CTL epitopes by assessing the ability to stimulate HTL responses and/or by using appropriate transgenic mouse models. Immunogenicity is determined by screening for: 1.) *in vitro* primary induction using normal PBMC or 2.) recall responses from cancer patient PBMCs.

#### Example 7. Calculation of phenotypic frequencies of HLA-supertypes in various ethnic backgrounds to determine breadth of population coverage

This example illustrates the assessment of the breadth of population coverage of a vaccine composition comprised of multiple epitopes comprising multiple supermotifs and/or motifs.

In order to analyze population coverage, gene frequencies of HLA alleles were determined. Gene frequencies for each HLA allele were calculated from antigen or allele frequencies utilizing the binomial distribution formulae  $gf=1-(\text{SQRT}(1-af))$  (see, e.g., Sidney *et al.*, *Human Immunol.* 45:79-93, 1996). To obtain overall phenotypic frequencies, cumulative gene frequencies were calculated, and the cumulative antigen frequencies derived by the use of the inverse formula  $[af=1-(1-Cgf)^2]$ .

Where frequency data was not available at the level of DNA typing, correspondence to the serologically defined antigen frequencies was assumed. To obtain total potential supertype population coverage no linkage disequilibrium was assumed, and only alleles confirmed to belong to each of the superotypes were included (minimal estimates). Estimates of total potential coverage achieved by inter-loci combinations were made by adding to the A coverage the proportion of the non-A covered population that could be expected to be covered by the B alleles considered (e.g.,  $\text{total}=A+B*(1-A)$ ). Confirmed members of the A3-like supertype are A3, A11, A31, A\*3301, and A\*6801. Although the A3-like supertype may also include A34, A66, and A\*7401, these alleles were not included in overall frequency calculations.

Likewise, confirmed members of the A2-like supertype family are A\*0201, A\*0202, A\*0203, A\*0204, A\*0205, A\*0206, A\*0207, A\*6802, and A\*6901. Finally, the B7-like supertype-confirmed alleles are: B7, B\*3501-03, B51, B\*5301, B\*5401, B\*5501-2, B\*5601, B\*6701, and B\*7801 (potentially also B\*1401, B\*3504-06, B\*4201, and B\*5602).

5 Population coverage achieved by combining the A2-, A3- and B7-supertypes is approximately 86% in five major ethnic groups, *supra*. Coverage may be extended by including peptides bearing the A1 and A24 motifs. On average, A1 is present in 12% and A24 in 29% of the population across five different major ethnic groups (Caucasian, North American Black, Chinese, Japanese, and Hispanic). Together, these alleles are represented with an average frequency of 39% in these same ethnic populations.

10 The total coverage across the major ethnicities when A1 and A24 are combined with the coverage of the A2-, A3- and B7-supertype alleles is >95%. An analogous approach can be used to estimate population coverage achieved with combinations of class II motif-bearing epitopes.

Immunogenicity studies in humans (*e.g.*, Bertoni *et al.*, *J. Clin. Invest.* 100:503, 1997; Doolan *et al.*, *Immunity* 7:97, 1997; and Threlkeld *et al.*, *J. Immunol.* 159:1648, 1997) have shown that

15 highly cross-reactive binding peptides are almost always recognized as epitopes. The use of highly cross-reactive binding peptides is an important selection criterion in identifying candidate epitopes for inclusion in a vaccine that is immunogenic in a diverse population.

With a sufficient number of epitopes (as disclosed herein and from the art), an average population coverage is predicted to be greater than 95% in each of five major ethnic populations. The game theory Monte Carlo simulation analysis, which is known in the art (see *e.g.*, Osborne, M.J. and Rubinstein, A. "A course in game theory" MIT Press, 1994), can be used to estimate what percentage of the individuals

20 in a population comprised of the Caucasian, North American Black, Japanese, Chinese, and Hispanic ethnic groups would recognize the vaccine epitopes described herein. A preferred percentage is 90%. A more preferred percentage is 95%.

25

#### Example 8. CTL Recognition Of Endogenous Processed Antigens After Priming

This example determines that CTL induced by native or analogued peptide epitopes identified and selected as described in Examples 1-6 recognize endogenously synthesized, *i.e.*, native antigens.

30 Effector cells isolated from transgenic mice that are immunized with peptide epitopes as in Example 3, for example HLA-A2 supermotif-bearing epitopes, are re-stimulated *in vitro* using peptide-coated stimulator cells. Six days later, effector cells are assayed for cytotoxicity and the cell lines that contain peptide-specific cytotoxic activity are further re-stimulated. An additional six days later, these cell lines are tested for cytotoxic activity on <sup>51</sup>Cr labeled Jurkat-A2.1/K<sup>b</sup> target cells in the absence or presence

35 of peptide, and also tested on <sup>51</sup>Cr labeled target cells bearing the endogenously synthesized antigen, *i.e.* cells that are stably transfected with HPV expression vectors.

The result will demonstrate that CTL lines obtained from animals primed with peptide epitope recognize endogenously synthesized HPV antigen. The choice of transgenic mouse model to be used for such an analysis depends upon the epitope(s) that is being evaluated. In addition to HLA-

40 A\*0201/K<sup>b</sup> transgenic mice, several other transgenic mouse models including mice with human A11, which

may also be used to evaluate A3 epitopes, and B7 alleles have been characterized and others (e.g., transgenic mice for HLA-A1 and A24) are being developed. HLA-DR1 and HLA-DR3 mouse models have also been developed, which may be used to evaluate HTL epitopes.

#### 5 Example 9. Activity Of CTL-HTL Conjugated Epitopes In Transgenic Mice

This example illustrates the induction of CTLs and HTLs in transgenic mice by use of a tumor associated antigen CTL/HTL peptide conjugate whereby the vaccine composition comprises peptides to be administered to an HPV-infected patient. The peptide composition can comprise multiple CTL and/or HTL epitopes and further, can comprise epitopes selected from multiple HPV target antigens. The epitopes are identified using methodology as described in Examples 1-6. This analysis demonstrates the enhanced immunogenicity that can be achieved by inclusion of one or more HTL epitopes in a vaccine composition. Such a peptide composition can comprise an HTL epitope conjugated to a preferred CTL epitope containing, for example, at least one CTL epitope that binds to multiple HLA family members at an affinity of 500 nM or less, or analogs of that epitope. The peptides may be lipidated, if desired.

Immunization procedures: Immunization of transgenic mice is performed as described (Alexander *et al.*, *J. Immunol.* 159:4753-4761, 1997). For example, A2/K<sup>b</sup> mice, which are transgenic for the human HLA A2.1 allele and are useful for the assessment of the immunogenicity of HLA-A\*0201 motif- or HLA-A2 supermotif-bearing epitopes, are primed subcutaneously (base of the tail) with a 0.1 ml of peptide in Incomplete Freund's Adjuvant, or if the peptide composition is a lipidated CTL/HTL conjugate, in DMSO/saline or if the peptide composition is a polypeptide, in PBS or Incomplete Freund's Adjuvant. Seven days after priming, splenocytes obtained from these animals are restimulated with syngenic irradiated LPS-activated lymphoblasts coated with peptide.

Cell lines: Target cells for peptide-specific cytotoxicity assays are Jurkat cells transfected with the HLA-A2.1/K<sup>b</sup> chimeric gene (e.g., Vitiello *et al.*, *J. Exp. Med.* 173:1007, 1991).

*In vitro* CTL activation: One week after priming, spleen cells ( $30 \times 10^6$  cells/flask) are co-cultured at 37°C with syngenic, irradiated (3000 rads), peptide coated lymphoblasts ( $10 \times 10^6$  cells/flask) in 10 ml of culture medium/T25 flask. After six days, effector cells are harvested and assayed for cytotoxic activity.

Assay for cytotoxic activity: Target cells ( $1.0$  to  $1.5 \times 10^6$ ) are incubated at 37°C in the presence of 200  $\mu$ l of  $^{51}\text{Cr}$ . After 60 minutes, cells are washed three times and resuspended in R10 medium. Peptide is added where required at a concentration of 1  $\mu$ g/ml. For the assay,  $10^4$   $^{51}\text{Cr}$ -labeled target cells are added to different concentrations of effector cells (final volume of 200  $\mu$ l) in U-bottom 96-well plates. After a 6 hour incubation period at 37°C, a 0.1 ml aliquot of supernatant is removed from each well and radioactivity is determined in a Micromedic automatic gamma counter. The percent specific lysis is determined by the formula: percent specific release =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . To facilitate comparison between separate CTL assays run under the same conditions, %  $^{51}\text{Cr}$  release data is expressed as lytic units/ $10^6$  cells. One lytic unit is arbitrarily defined as the number of effector cells required to achieve 30% lysis of 10,000 target cells in a 6 hour  $^{51}\text{Cr}$  release assay. To obtain specific lytic units/ $10^6$ , the lytic units/ $10^6$  obtained in the absence of peptide is subtracted from the lytic units/ $10^6$  obtained in the presence of peptide. For example, if 30%  $^{51}\text{Cr}$

release is obtained at the effector (E): target (T) ratio of 50:1 (i.e.,  $5 \times 10^5$  effector cells for 10,000 targets) in the absence of peptide and 5:1 (i.e.,  $5 \times 10^4$  effector cells for 10,000 targets) in the presence of peptide, the specific lytic units would be:  $[(1/50,000)-(1/500,000)] \times 10^6 = 18 \text{ LU}$ .

5 The results are analyzed to assess the magnitude of the CTL responses of animals injected with the immunogenic CTL/HTL conjugate vaccine preparation and are compared to the magnitude of the CTL response achieved using the CTL epitope as outlined in Example 3. Analyses similar to this may be performed to evaluate the immunogenicity of peptide conjugates containing multiple CTL epitopes and/or multiple HTL epitopes. In accordance with these procedures it is found that a CTL response is induced, and concomitantly that an HTL response is induced upon administration of such compositions.

10

Example 10. Selection of CTL and HTL epitopes for inclusion in an HPV-specific vaccine.

This example illustrates the procedure for the selection of peptide epitopes for vaccine compositions of the invention. The peptides in the composition can be in the form of a nucleic acid sequence, either single or one or more sequences (i.e., minigene) that encodes peptide(s), or can be single and/or polypeptidic peptides.

15

The following principles are utilized when selecting an array of epitopes for inclusion in a vaccine composition. Each of the following principles is balanced in order to make the selection.

20

Epitopes are selected which, upon administration, mimic immune responses that have been observed to be correlated with HPV clearance. The number of epitopes used depends on observations of patients who spontaneously clear HPV. For example, if it has been observed that patients who spontaneously clear HPV generate an immune response to at least 3 epitopes on at least one HPV antigen, then 3-4 epitopes should be included for HLA class I. A similar rationale is used to determine HLA class II epitopes.

25

When selecting an array of HPV epitopes, it is preferred that at least some of the epitopes are derived from early and late proteins. The early proteins of HPV are expressed when the virus is replicating, either following acute or dormant infection. Therefore, it is particularly preferred to use epitopes from early stage proteins to alleviate disease manifestations at the earliest stage possible.

30

Epitopes are often selected that have a binding affinity of an  $IC_{50}$  of 500 nM or less for an HLA class I molecule, or for class II, an  $IC_{50}$  of 1000 nM or less.

Sufficient supermotif bearing peptides, or a sufficient array of allele-specific motif bearing peptides, are selected to give broad population coverage. For example, epitopes are selected to provide at least 80% population coverage. A Monte Carlo analysis, a statistical evaluation known in the art, can be employed to assess breadth, or redundancy, of population coverage.

35

When creating a polypeptidic compositions, e.g. a minigene, it is typically desirable to generate the smallest peptide possible that encompasses the epitopes of interest. The principles employed are similar, if not the same, as those employed when selecting a peptide comprising nested epitopes.

In cases where the sequences of multiple variants of the same target protein are available, potential peptide epitopes can also be selected on the basis of their conservancy. For example, a criterion for conservancy may define that the entire sequence of an HLA class I binding peptide or the entire 9-mer

core of a class II binding peptide be conserved in a designated percentage of the sequences evaluated for a specific protein antigen.

5 A vaccine composition comprised of selected peptides, when administered, is safe, efficacious, and elicits an immune response similar in magnitude to an immune response that controls or clears an acute HPV infection.

Example 11. Construction of Minigene Multi-Epitope DNA Plasmids

10 This example provides general guidance for the construction of a minigene expression plasmid. Minigene plasmids may, of course, contain various configurations of CTL and/or HTL epitopes or epitope analogs as described herein. Examples of the construction and evaluation of expression plasmids are described, for example, in co-pending U.S.S.N. 09/311,784 filed 5/13/99.

15 A minigene expression plasmid typically includes multiple CTL and HTL peptide epitopes. In the present example, HLA-A2, -A3, -B7 supermotif-bearing peptide epitopes and HLA-A1 and -A24 motif-bearing peptide epitopes are used in conjunction with DR supermotif-bearing epitopes and/or DR3 epitopes. HLA class I supermotif or motif-bearing peptide epitopes derived from multiple HPV antigens, preferably including both early and late phase antigens, are selected such that multiple supermotifs/motifs are represented to ensure broad population coverage. Similarly, HLA class II epitopes are selected from multiple HPV antigens to provide broad population coverage, *i.e.* both HLA DR-1-4-7 supermotif-bearing epitopes and HLA DR-3 motif-bearing epitopes are selected for inclusion in the minigene construct. The selected CTL and HTL epitopes are then incorporated into a minigene for expression in an expression vector.

20 Such a construct may additionally include sequences that direct the HTL epitopes to the endoplasmic reticulum. For example, the Ii protein may be fused to one or more HTL epitopes as described in co-pending application U.S.S.N. 09/311,784 filed 5/13/99, wherein the CLIP sequence of the Ii protein is removed and replaced with an HLA class II epitope sequence so that HLA class II epitope is directed to the endoplasmic reticulum, where the epitope binds to an HLA class II molecules.

25 This example illustrates the methods to be used for construction of a minigene-bearing expression plasmid. Other expression vectors that may be used for minigene compositions are available and known to those of skill in the art.

30 The minigene DNA plasmid of this example contains a consensus Kozak sequence and a consensus murine kappa Ig-light chain signal sequence followed by CTL and/or HTL epitopes selected in accordance with principles disclosed herein. The sequence encodes an open reading frame fused to the Myc and His antibody epitope tag coded for by the pcDNA 3.1 Myc-His vector.

35 Overlapping oligonucleotides that can, for example, average about 70 nucleotides in length with 15 nucleotide overlaps, are synthesized and HPLC-purified. The oligonucleotides encode the selected peptide epitopes as well as appropriate linker nucleotides, Kozak sequence, and signal sequence. The final multi-epitope minigene is assembled by extending the overlapping oligonucleotides in three sets of reactions using PCR. A Perkin/Elmer 9600 PCR machine is used and a total of 30 cycles are performed using the following conditions: 95°C for 15 sec, annealing temperature (5° below the lowest calculated T<sub>m</sub> of each primer pair) for 30 sec, and 72°C for 1 min.



For example, a minigene can be prepared as follows. For a first PCR reaction, 5 µg of each of two oligonucleotides are annealed and extended: In an example using eight oligonucleotides, i.e., four pairs of primers, oligonucleotides 1+2, 3+4, 5+6, and 7+8 are combined in 100 µl reactions containing *Pfu* polymerase buffer (1x= 10 mM KCL, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-chloride, pH 8.75, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 100 µg/ml BSA), 0.25 mM each dNTP, and 2.5 U of *Pfu* polymerase. The full-length dimer products are gel-purified, and two reactions containing the product of 1+2 and 3+4, and the product of 5+6 and 7+8 are mixed, annealed, and extended for 10 cycles. Half of the two reactions are then mixed, and 5 cycles of annealing and extension carried out before flanking primers are added to amplify the full length product. The full-length product is gel-purified and cloned into pCR-blunt (Invitrogen) and individual clones are screened by sequencing.

Example 12. The plasmid construct and the degree to which it induces immunogenicity.

The degree to which a plasmid construct, for example a plasmid constructed in accordance with Example 11, is able to induce immunogenicity can be evaluated *in vitro* by testing for epitope presentation by APC following transduction or transfection of the APC with an epitope-expressing nucleic acid construct. Such a study determines "antigenicity" and allows the use of human APC. The assay determines the ability of the epitope to be presented by the APC in a context that is recognized by a T cell by quantifying the density of epitope-HLA class I complexes on the cell surface. Quantitation can be performed by directly measuring the amount of peptide eluted from the APC (see, e.g., Sijts *et al.*, *J. Immunol.* 156:683-692, 1996; Demotz *et al.*, *Nature* 342:682-684, 1989); or the number of peptide-HLA class I complexes can be estimated by measuring the amount of lysis or lymphokine release induced by infected or transfected target cells, and then determining the concentration of peptide necessary to obtained equivalent levels of lysis or lymphokine release (see, e.g., Kageyama *et al.*, *J. Immunol.* 154:567-576, 1995).

Alternatively, immunogenicity can be evaluated through *in vivo* injections into mice and subsequent *in vitro* assessment of CTL and HTL activity, which are analysed using cytotoxicity and proliferation assays, respectively, as detailed e.g., in copending U.S.S.N. 09/311,784 filed 5/13/99 and Alexander *et al.*, *Immunity* 1:751-761, 1994.

For example, to assess the capacity of a DNA minigene construct (e.g., a pMin minigene construct generated as described in U.S.S.N. 09/311,784) containing at least one HLA-A2 supermotif peptide to induce CTLs *in vivo*, HLA-A2.1/K<sup>b</sup> transgenic mice, for example, are immunized intramuscularly with 100 µg of naked cDNA. As a means of comparing the level of CTLs induced by cDNA immunization, a control group of animals is also immunized with an actual peptide composition that comprises multiple epitopes synthesized as a single polypeptide as they would be encoded by the minigene.

Splenocytes from immunized animals are stimulated twice with each of the respective compositions (peptide epitopes encoded in the minigene or the polyepitopic peptide), then assayed for peptide-specific cytotoxic activity in a <sup>51</sup>Cr release assay. The results indicate the magnitude of the CTL response directed against the A2-restricted epitope, thus indicating the *in vivo* immunogenicity of the minigene vaccine and polyepitopic vaccine. It is, therefore, found that the minigene elicits immune responses directed toward the HLA-A2 supermotif peptide epitopes as does the polyepitopic peptide

vaccine. A similar analysis is also performed using other HLA-A3 and HLA-B7 transgenic mouse models to assess CTL induction by HLA-A3 and HLA-B7 motif or supermotif epitopes.

To assess the capacity of a class II epitope encoding minigene to induce HTLs *in vivo*, DR transgenic mice, or for those epitope that cross react with the appropriate mouse MHC molecule, I-A<sup>b</sup>-restricted mice, for example, are immunized intramuscularly with 100 µg of plasmid DNA. As a means of comparing the level of HTLs induced by DNA immunization, a group of control animals is also immunized with an actual peptide composition emulsified in complete Freund's adjuvant. CD4<sup>+</sup> T cells, *i.e.* HTLs, are purified from splenocytes of immunized animals and stimulated with each of the respective compositions (peptides encoded in the minigene). The HTL response is measured using a <sup>3</sup>H-thymidine incorporation proliferation assay, (*see, e.g.*, Alexander et al. *Immunity* 1:751-761, 1994). The results indicate the magnitude of the HTL response, thus demonstrating the *in vivo* immunogenicity of the minigene.

DNA minigenes, constructed as described in Example 11, may also be evaluated as a vaccine in combination with a boosting agent using a prime boost protocol. The boosting agent can consist of recombinant protein (*e.g.*, Barnett et al., *Aids Res. and Human Retroviruses* 14, Supplement 3:S299-S309, 1998) or recombinant vaccinia, for example, expressing a minigene or DNA encoding the complete protein of interest (*see, e.g.*, Hanke et al., *Vaccine* 16:439-445, 1998; Sedegah et al., *Proc. Natl. Acad. Sci USA* 95:7648-53, 1998; Hanke and McMichael, *Immunol. Letters* 66:177-181, 1999; and Robinson et al., *Nature Med.* 5:526-34, 1999).

For example, the efficacy of the DNA minigene used in a prime boost protocol is initially evaluated in transgenic mice. In this example, A2.1/K<sup>b</sup> transgenic mice are immunized IM with 100 µg of a DNA minigene encoding the immunogenic peptides including at least one HLA-A2 supermotif-bearing peptide. After an incubation period (ranging from 3-9 weeks), the mice are boosted IP with 10<sup>7</sup> pfu/mouse of a recombinant vaccinia virus expressing the same sequence encoded by the DNA minigene. Control mice are immunized with 100 µg of DNA or recombinant vaccinia without the minigene sequence, or with DNA encoding the minigene, but without the vaccinia boost. After an additional incubation period of two weeks, splenocytes from the mice are immediately assayed for peptide-specific activity in an ELISPOT assay. Additionally, splenocytes are stimulated *in vitro* with the A2-restricted peptide epitopes encoded in the minigene and recombinant vaccinia, then assayed for peptide-specific activity in an IFN-γ ELISA.

It is found that the minigene utilized in a prime-boost protocol elicits greater immune responses toward the HLA-A2 supermotif peptides than with DNA alone. Such an analysis can also be performed using HLA-A11 or HLA-B7 transgenic mouse models to assess CTL induction by HLA-A3 or HLA-B7 motif or supermotif epitopes.

The use of prime boost protocols in humans is described in Example 20.

### 35 Example 13. Peptide Composition for Prophylactic Uses

Vaccine compositions of the present invention can be used to prevent HPV infection in persons who are at risk for such infection. For example, a polypeptidic peptide epitope composition (or a nucleic acid comprising the same) containing multiple CTL and HTL epitopes such as those selected in Examples 9 and/or 10, which are also selected to target greater than 80% of the population, is administered to individuals at risk for HPV infection.

For example, a peptide-based composition can be provided as a single polypeptide that encompasses multiple epitopes. The vaccine is typically administered in a physiological solution that comprises an adjuvant, such as Incomplete Freund's Adjuvant. The dose of peptide for the initial immunization is from about 1 to about 50,000 µg, generally 100-5,000 µg, for a 70 kg patient. The initial administration of vaccine is followed by booster dosages at 4 weeks followed by evaluation of the magnitude of the immune response in the patient, by techniques that determine the presence of epitope-specific CTL populations in a PBMC sample. Additional booster doses are administered as required. The composition is found to be both safe and efficacious as a prophylaxis against HPV infection.

Alternatively, a composition typically comprising transfecting agents can be used for the administration of a nucleic acid-based vaccine in accordance with methodologies known in the art and disclosed herein.

#### Example 14. Polyepitopic Vaccine Compositions Derived from Native HPV Sequences

A native HPV polyprotein sequence is screened, preferably using computer algorithms defined for each class I and/or class II supermotif or motif, to identify "relatively short" regions of the polyprotein that comprise multiple epitopes and is preferably less in length than an entire native antigen. This relatively short sequence that contains multiple distinct, even overlapping, epitopes is selected and used to generate a minigene construct. The construct is engineered to express the peptide, which corresponds to the native protein sequence. The "relatively short" peptide is generally less than 250 amino acids in length, often less than 100 amino acids in length, preferably less than 75 amino acids in length, and more preferably less than 50 amino acids in length. The protein sequence of the vaccine composition is selected because it has maximal number of epitopes contained within the sequence, *i.e.*, it has a high concentration of epitopes. As noted herein, epitope motifs may be nested or overlapping (*i.e.*, frame shifted relative to one another). For example, with *f* overlapping epitopes, two 9-mer epitopes and one 10-mer epitope can be present in a 10 amino acid peptide. Such a vaccine composition is administered for therapeutic or prophylactic purposes.

The vaccine composition will include, for example, three CTL epitopes from at least one HPV target antigen and at least one HTL epitope. This polyepitopic native sequence is administered either as a peptide or as a nucleic acid sequence which encodes the peptide. Alternatively, an analog can be made of this native sequence, whereby one or more of the epitopes comprise substitutions that alter the cross-reactivity and/or binding affinity properties of the polyepitopic peptide.

The embodiment of this example provides for the possibility that an as yet undiscovered aspect of immune system processing will apply to the native nested sequence and thereby facilitate the production of therapeutic or prophylactic immune response-inducing vaccine compositions. Additionally such an embodiment provides for the possibility of motif-bearing epitopes for an HLA makeup that is presently unknown. Furthermore, this embodiment (absent analogs) directs the immune response to multiple peptide sequences that are actually present in native HPV antigens thus avoiding the need to evaluate any junctional epitopes. Lastly, the embodiment provides an economy of scale when producing nucleic acid vaccine compositions.

Related to this embodiment, computer programs can be derived in accordance with principles in the art, which identify in a target sequence, the greatest number of epitopes per sequence length.

5 Example 15. Polyepitopic Vaccine Compositions From Multiple Antigens

The HPV peptide epitopes of the present invention are used in conjunction with peptide epitopes from other target tumor-associated antigens to create a vaccine composition that is useful for the prevention or treatment of cancer resulting from HPV infection in multiple patients.

For example, a vaccine composition can be provided as a single polypeptide that  
10 incorporates multiple epitopes from HPV antigens as well as tumor-associated antigens that are often expressed with a target cancer, e.g., cervical cancer, associated with HPV infection, or can be administered as a composition comprising one or more discrete epitopes. Alternatively, the vaccine can be administered as a minigene construct or as dendritic cells which have been loaded with the peptide epitopes *in vitro*.

15 Example 16. Use of peptides to evaluate an immune response

Peptides of the invention may be used to analyze an immune response for the presence of specific CTL or HTL populations directed to HPV. Such an analysis may be performed in a manner as that described by Ogg *et al.*, *Science* 279:2103-2106, 1998. In the following example, peptides in accordance with the invention are used as a reagent for diagnostic or prognostic purposes, not as an immunogen.

In this example highly sensitive human leukocyte antigen tetrameric complexes  
20 ("tetramers") are used for a cross-sectional analysis of, for example, HPV HLA-A\*0201-specific CTL frequencies from HLA A\*0201-positive individuals at different stages of infection or following immunization using an HPV peptide containing an A\*0201 motif. Tetrameric complexes are synthesized as described (Musey *et al.*, *N. Engl. J. Med.* 337:1267, 1997). Briefly, purified HLA heavy chain (A\*0201 in  
25 this example) and  $\beta$ 2-microglobulin are synthesized by means of a prokaryotic expression system. The heavy chain is modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a sequence containing a BirA enzymatic biotinylation site. The heavy chain,  $\beta$ 2-microglobulin, and peptide are refolded by dilution. The 45-kD refolded product is isolated by fast protein liquid chromatography and then biotinylated by BirA in the presence of biotin (Sigma, St. Louis, Missouri), adenosine 5'triphosphate  
30 and magnesium. Streptavidin-phycoerythrin conjugate is added in a 1:4 molar ratio, and the tetrameric product is concentrated to 1 mg/ml. The resulting product is referred to as tetramer-phycoerythrin.

For the analysis of patient blood samples, approximately one million PBMCs are centrifuged at 300g for 5 minutes and resuspended in 50  $\mu$ l of cold phosphate-buffered saline. Tri-color analysis is performed with the tetramer-phycoerythrin, along with anti-CD8-Tricolor, and anti-CD38. The  
35 PBMCs are incubated with tetramer and antibodies on ice for 30 to 60 min and then washed twice before formaldehyde fixation. Gates are applied to contain >99.98% of control samples. Controls for the tetramers include both A\*0201-negative individuals and A\*0201-positive uninfected donors. The percentage of cells stained with the tetramer is then determined by flow cytometry. The results indicate the number of cells in the PBMC sample that contain epitope-restricted CTLs, thereby readily indicating the

extent of immune response to the HPV epitope, and thus the stage of infection with HPV, the status of exposure to HPV, or exposure to a vaccine that elicits a protective or therapeutic response.

Example 17. Use of Peptide Epitopes to Evaluate Recall Responses

5           The peptide epitopes of the invention are used as reagents to evaluate T cell responses, such as acute or recall responses, in patients. Such an analysis may be performed on patients who have recovered from infection, who are chronically infected with HPV, or who have been vaccinated with an HPV vaccine.

10           For example, the class I restricted CTL response of persons who have been vaccinated may be analyzed. The vaccine may be any HPV vaccine. PBMC are collected from vaccinated individuals and HLA typed. Appropriate peptide epitopes of the invention that, optimally, bear supermotifs to provide cross-reactivity with multiple HLA supertype family members, are then used for analysis of samples derived from individuals who bear that HLA type.

15           PBMC from vaccinated individuals are separated on Ficoll-Histopaque density gradients (Sigma Chemical Co., St. Louis, MO), washed three times in HBSS (GIBCO Laboratories), resuspended in RPMI-1640 (GIBCO Laboratories) supplemented with L-glutamine (2mM), penicillin (50U/ml), streptomycin (50 µg/ml), and Hepes (10mM) containing 10% heat-inactivated human AB serum (complete RPMI) and plated using microculture formats. A synthetic peptide comprising an epitope of the invention is added at 10 µg/ml to each well and HBV core 128-140 epitope is added at 1 µg/ml to each well as a  
20           source of T cell help during the first week of stimulation.

          In the microculture format,  $4 \times 10^5$  PBMC are stimulated with peptide in 8 replicate cultures in 96-well round bottom plate in 100 µl/well of complete RPMI. On days 3 and 10, 100 ul of complete RPMI and 20 U/ml final concentration of rIL-2 are added to each well. On day 7 the cultures are transferred into a 96-well flat-bottom plate and restimulated with peptide, rIL-2 and  $10^5$  irradiated (3,000  
25           rad) autologous feeder cells. The cultures are tested for cytotoxic activity on day 14. A positive CTL response requires two or more of the eight replicate cultures to display greater than 10% specific  $^{51}\text{Cr}$  release, based on comparison with uninfected control subjects as previously described (Rehermann, *et al.*, *Nature Med.* 2:1104,1108, 1996; Rehermann *et al.*, *J. Clin. Invest.* 97:1655-1665, 1996; and Rehermann *et al.* *J. Clin. Invest.* 98:1432-1440, 1996).

30           Target cell lines are autologous and allogeneic EBV-transformed B-LCL that are either purchased from the American Society for Histocompatibility and Immunogenetics (ASHI, Boston, MA) or established from the pool of patients as described (Guilhot, *et al.* *J. Virol.* 66:2670-2678, 1992).

          Cytotoxicity assays are performed in the following manner. Target cells consist of either allogeneic HLA-matched or autologous EBV-transformed B lymphoblastoid cell line that are incubated  
35           overnight with the synthetic peptide epitope of the invention at 10 µM, and labeled with 100 µCi of  $^{51}\text{Cr}$  (Amersham Corp., Arlington Heights, IL) for 1 hour after which they are washed four times with HBSS.

          Cytolytic activity is determined in a standard 4-h, split well  $^{51}\text{Cr}$  release assay using U-bottomed 96 well plates containing 3,000 targets/well. Stimulated PBMC are tested at effector/target (E/T) ratios of 20-50:1 on day 14. Percent cytotoxicity is determined from the formula:  $100 \times [( \text{experimental release} - \text{spontaneous release} ) / \text{maximum release} - \text{spontaneous release} ]$ . Maximum release is determined by  
40

lysis of targets by detergent (2% Triton X-100; Sigma Chemical Co., St. Louis, MO). Spontaneous release is <25% of maximum release for all experiments.

The results of such an analysis indicate the extent to which HLA-restricted CTL populations have been stimulated by previous exposure to HPV or an HPV vaccine.

5 The class II restricted HTL responses may also be analyzed. Purified PBMC are cultured in a 96-well flat bottom plate at a density of  $1.5 \times 10^5$  cells/well and are stimulated with 10  $\mu$ g/ml synthetic peptide, whole antigen, or PHA. Cells are routinely plated in replicates of 4-6 wells for each condition. After seven days of culture, the medium is removed and replaced with fresh medium containing 10U/ml IL-2. Two days later, 1  $\mu$ Ci  $^3$ H-thymidine is added to each well and incubation is continued for an additional 10 18 hours. Cellular DNA is then harvested on glass fiber mats and analyzed for  $^3$ H-thymidine incorporation. Antigen-specific T cell proliferation is calculated as the ratio of  $^3$ H-thymidine incorporation in the presence of antigen divided by the  $^3$ H-thymidine incorporation in the absence of antigen.

#### Example 18. Induction Of Specific CTL Response In Humans

15 A human clinical trial for an immunogenic composition comprising CTL and HTL epitopes of the invention is set up as an IND Phase I, dose escalation study and carried out as a randomized, double-blind, placebo-controlled trial. Such a trial is designed, for example, as follows:

A total of about 27 individuals are enrolled and divided into 3 groups:

20 Group I: 3 subjects are injected with placebo and 6 subjects are injected with 5  $\mu$ g of peptide composition;

Group II: 3 subjects are injected with placebo and 6 subjects are injected with 50  $\mu$ g peptide composition;

Group III: 3 subjects are injected with placebo and 6 subjects are injected with 500  $\mu$ g of peptide composition.

25 After 4 weeks following the first injection, all subjects receive a booster inoculation at the same dosage.

30 The endpoints measured in this study relate to the safety and tolerability of the peptide composition as well as its immunogenicity. Cellular immune responses to the peptide composition are an index of the intrinsic activity of this the peptide composition, and can therefore be viewed as a measure of biological efficacy. The following summarize the clinical and laboratory data that relate to safety and efficacy endpoints.

Safety: The incidence of adverse events is monitored in the placebo and drug treatment group and assessed in terms of degree and reversibility.

35 Evaluation of Vaccine Efficacy: For evaluation of vaccine efficacy, subjects are bled before and after injection. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation, aliquoted in freezing media and stored frozen. Samples are assayed for CTL and HTL activity.

The vaccine is found to be both safe and efficacious.

Example 19. Phase II Trials In Patients Infected With HPV

Phase II trials are performed to study the effect of administering the CTL-HTL peptide compositions to patients having cancer associated with HPV infection. The main objectives of the trials are to determine an effective dose and regimen for inducing CTLs in HPV-infected patients with cancer, to establish the safety of inducing a CTL and HTL response in these patients, and to see to what extent activation of CTLs improves the clinical picture of chronically infected HPV patients, as manifested by a reduction in viral load, e.g., the reduction and/or shrinking of lesions. Such a study is designed, for example, as follows:

The studies are performed in multiple centers. The trial design is an open-label, uncontrolled, dose escalation protocol wherein the peptide composition is administered as a single dose followed six weeks later by a single booster shot of the same dose. The dosages are 50, 500 and 5,000 micrograms per injection. Drug-associated adverse effects (severity and reversibility) are recorded.

There are three patient groupings. The first group is injected with 50 micrograms of the peptide composition and the second and third groups with 500 and 5,000 micrograms of peptide composition, respectively. The patients within each group range in age from 21-65 and represent diverse ethnic backgrounds. All of them are infected with HPV and are HIV, HCV, HBV and delta hepatitis virus (HDV) negative, but are positive for HPV DNA as monitored by PCR.

Clinical manifestations or antigen-specific T-cell responses are monitored to assess the effects of administering the peptide compositions. The vaccine composition is found to be both safe and efficacious in the treatment of HPV infection.

Example 20. Induction of CTL Responses Using a Prime Boost Protocol

A prime boost protocol similar in its underlying principle to that used to evaluate the efficacy of a DNA vaccine in transgenic mice, such as described in Example 12, can also be used for the administration of the vaccine to humans. Such a vaccine regimen can include an initial administration of, for example, naked DNA followed by a boost using recombinant virus encoding the vaccine, or recombinant protein/polypeptide or a peptide mixture administered in an adjuvant.

For example, the initial immunization may be performed using an expression vector, such as that constructed in Example 11, in the form of naked nucleic acid administered IM (or SC or ID) in the amounts of 0.5-5 mg at multiple sites. The nucleic acid (0.1 to 1000 µg) can also be administered using a gene gun. Following an incubation period of 3-4 weeks, a booster dose is then administered. The booster can be recombinant fowlpox virus administered at a dose of  $5 \cdot 10^7$  to  $5 \cdot 10^9$  pfu. An alternative recombinant virus, such as an MVA, canarypox, adenovirus, or adeno-associated virus, can also be used for the booster, or the polypeptidic protein or a mixture of the peptides can be administered. For evaluation of vaccine efficacy, patient blood samples will be obtained before immunization as well as at intervals following administration of the initial vaccine and booster doses of the vaccine. Peripheral blood mononuclear cells are isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation, aliquoted in freezing media and stored frozen. Samples are assayed for CTL and HTL activity.

Analysis of the results indicates that a magnitude of response sufficient to achieve protective immunity against HPV is generated.

Example 21. Administration of Vaccine Compositions Using Dendritic Cells (DC)

Vaccines comprising peptide epitopes of the invention can be administered using APCs, or "professional" APCs such as DC. In this example, the peptide-pulsed DC are administered to a patient to stimulate a CTL response *in vivo*. In this method, dendritic cells are isolated, expanded, and pulsed with a vaccine comprising peptide CTL and HTL epitopes of the invention. The dendritic cells are infused back into the patient to elicit CTL and HTL responses *in vivo*. The induced CTL and HTL then destroy or facilitate destruction of the specific target cells that bear the proteins from which the epitopes in the vaccine are derived.

For example, a cocktail of epitope-bearing peptides is administered *ex vivo* to PBMC, or isolated DC therefrom. A pharmaceutical to facilitate harvesting of DC can be used, such as Progenipoiectin (Monsanto, St. Louis, MO) or GM-CSF/IL-4. After pulsing the DC with peptides and prior to reinfusion into patients, the DC are washed to remove unbound peptides.

As appreciated clinically, and readily determined by one of skill based on clinical outcomes, the number of DC reinfused into the patient can vary (see, e.g., *Nature Med.* 4:328, 1998; *Nature Med.* 2:52, 1996 and *Prostate* 32:272, 1997). Although  $2-50 \times 10^6$  DC per patient are typically administered, larger number of DC, such as  $10^7$  or  $10^8$  can also be provided. Such cell populations typically contain between 50-90% DC.

In some embodiments, peptide-loaded PBMC are injected into patients without purification of the DC. For example, PBMC containing DC generated after treatment with an agent such as Progenipoiectin are injected into patients without purification of the DC. The total number of PBMC that are administered often ranges from  $10^8$  to  $10^{10}$ . Generally, the cell doses injected into patients is based on the percentage of DC in the blood of each patient, as determined, for example, by immunofluorescence analysis with specific anti-DC antibodies. Thus, for example, if Progenipoiectin™ mobilizes 2% DC in the peripheral blood of a given patient, and that patient is to receive  $5 \times 10^6$  DC, then the patient will be injected with a total of  $2.5 \times 10^8$  peptide-loaded PBMC. The percent DC mobilized by an agent such as Progenipoiectin™ is typically estimated to be between 2-10%, but can vary as appreciated by one of skill in the art.

Ex vivo activation of CTL/HTL responses

Alternatively, *ex vivo* CTL or HTL responses to HPV antigens can be induced by incubating in tissue culture the patient's, or genetically compatible, CTL or HTL precursor cells together with a source of APC, such as DC, and the appropriate immunogenic peptides. After an appropriate incubation time (typically about 7-28 days), in which the precursor cells are activated and expanded into effector cells, the cells are infused back into the patient, where they will destroy (CTL) or facilitate destruction (HTL) of their specific target cells, i.e., tumor cells.

Example 22. Alternative Method of Identifying Motif-Bearing Peptides

Another method of identifying motif-bearing peptides is to elute them from cells bearing defined MHC molecules. For example, EBV transformed B cell lines used for tissue typing have been



extensively characterized to determine which HLA molecules they express. In certain cases these cells express only a single type of HLA molecule. These cells can be infected with a pathogenic organism or transfected with nucleic acids that express the antigen of interest, *e.g.* HPV regulatory or structural proteins. Peptides produced by endogenous antigen processing of peptides produced consequent to infection (or as a result of transfection) will then bind to HLA molecules within the cell and be transported and displayed on the cell surface. Peptides are then eluted from the HLA molecules by exposure to mild acid conditions and their amino acid sequence determined, *e.g.*, by mass spectral analysis (*e.g.*, Kubo *et al.*, *J. Immunol.* 152:3913, 1994). Because the majority of peptides that bind a particular HLA molecule are motif-bearing, this is an alternative modality for obtaining the motif-bearing peptides correlated with the particular HLA molecule expressed on the cell.

Alternatively, cell lines that do not express endogenous HLA molecules can be transfected with an expression construct encoding a single HLA allele. These cells can then be used as described, *i.e.*, they can be infected with a pathogen or transfected with nucleic acid encoding an antigen of interest to isolate peptides corresponding to the pathogen or antigen of interest that have been presented on the cell surface. Peptides obtained from such an analysis will bear motif(s) that correspond to binding to the single HLA allele that is expressed in the cell.

As appreciated by one in the art, one can perform a similar analysis on a cell bearing more than one HLA allele and subsequently determine peptides specific for each HLA allele expressed. Moreover, one of skill would also recognize that means other than infection or transfection, such as loading with a protein antigen, can be used to provide a source of antigen to the cell.

The above examples are provided to illustrate the invention but not to limit its scope. For example, the human terminology for the Major Histocompatibility Complex, namely HLA, is used throughout this document. It is to be appreciated that these principles can be extended to other species as well. Thus, other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent application cited herein are hereby incorporated by reference for all purposes.

TABLE I

SUPERMOTIFS	POSITION	POSITION	POSITION
	2 (Primary Anchor)	3 (Primary Anchor)	C Terminus (Primary Anchor)
A1	<b>T</b> , <i>I, L, V, M, S</i>		<b>F, W, Y</b>
A2	<b>L</b> , <i>I, V, M, A, T, Q</i>		<b>I, V, M, A, T, L</b>
A3	<b>V, S, M, A, T, L, I</b>		<b>R, K</b>
A24	<b>Y, F, W, I, V, L, M, T</b>		<b>F, I, Y, W, L, M</b>
B7	<b>P</b>		<b>V, I, L, F, M, W, Y, A</b>
B27	<b>R, H, K</b>		<b>F, Y, L, W, M, I, V, A</b>
B44	<b>E, D</b>		<b>F, W, L, I, M, V, A</b>
B58	<b>A, T, S</b>		<b>F, W, Y, L, I, V, M, A</b>
B62	<b>Q, L, I, V, M, P</b>		<b>F, W, Y, M, I, V, L, A</b>
MOTIFS			
A1	<b>T, S, M</b>		<b>Y</b>
A1		<b>D, E, A, S</b>	<b>Y</b>
A2.1	<b>L, M, V, Q, I, A, T</b>		<b>V, L, I, M, A, T</b>
A3	<b>L, M, V, I, S, A, T, F, C, G, D</b>		<b>K, Y, R, H, F, A</b>
A11	<b>V, T, M, L, I, S, A, G, N, C, D, F</b>		<b>K, R, Y, H</b>
A24	<b>Y, F, W, M</b>		<b>F, L, I, W</b>
A*3101	<b>M, V, T, A, L, I, S</b>		<b>R, K</b>
A*3301	<b>M, V, A, L, F, I, S, T</b>		<b>R, K</b>
A*6801	<b>A, V, T, M, S, L, I</b>		<b>R, K</b>
B*0702	<b>P</b>		<b>L, M, F, W, Y, A, I, V</b>
B*3501	<b>P</b>		<b>L, M, F, W, Y, I, V, A</b>
B51	<b>P</b>		<b>L, I, V, F, W, Y, A, M</b>
B*5301	<b>P</b>		<b>I, M, F, W, Y, A, L, V</b>
B*5401	<b>P</b>		<b>A, T, I, V, L, M, F, W, Y</b>

Bolded residues are preferred, italicized residues are less preferred: A peptide is considered motif-bearing if it has primary anchors at each primary anchor position for a motif or supermotif as specified in the above table.

TABLE Ia

SUPERMOTIFS	POSITION	POSITION	POSITION
	2 (Primary Anchor)	3 (Primary Anchor)	C Terminus (Primary Anchor)
A1	<b>T, I, L, V, M, S</b>		<b>F, W, Y</b>
A2	<b>V, Q, A, T</b>		<b>I, V, L, M, A, T</b>
A3	<b>V, S, M, A, T, L, I</b>		<b>R, K</b>
A24	<b>Y, F, W, I, V, L, M, T</b>		<b>F, I, Y, W, L, M</b>
B7	<b>P</b>		<b>V, I, L, F, M, W, Y, A</b>
B27	<b>R, H, K</b>		<b>F, Y, L, W, M, I, V, A</b>
B58	<b>A, T, S</b>		<b>F, W, Y, L, I, V, M, A</b>
B62	<b>Q, L, I, V, M, P</b>		<b>F, W, Y, M, I, V, L, A</b>
MOTIFS			
A1	<b>T, S, M</b>		<b>Y</b>
A1		<b>D, E, A, S</b>	<b>Y</b>
A2.1	<b>V, Q, A, T*</b>		<b>V, L, I, M, A, T</b>
A3.2	<b>L, M, V, I, S, A, T, F, C, G, D</b>		<b>K, Y, R, H, F, A</b>
A11	<b>V, T, M, L, I, S, A, G, N, C, D, F</b>		<b>K, R, H, Y</b>
A24	<b>Y, F, W</b>		<b>F, L, I, W</b>

\*If 2 is V, or Q, the C-term is not L

Bolded residues are preferred, italicized residues are less preferred: A peptide is considered motif-bearing if it has primary anchors at each primary anchor position for a motif or supermotif as specified in the above table.

TABLE II

SUPERMOTIFS	POSITION							
	1	2	3	4	5	6	7	8 C-terminus
A1*		1° Anchor T,I,L,V,M,S						1° Anchor F,W,Y
A2		1° Anchor L,I,V,M,A, T,Q						1° Anchor L,I,V,M,A,T
A3	preferred	1° Anchor V,S,M,A,T; L,I	Y,F,W, (4/5)		Y,F,W, (3/5)	Y,F,W, (4/5)	P, (4/5)	1° Anchor R,K
	deleterious	D,E (3/5); P, (5/5)		D,E, (4/5)				
A24		1° Anchor Y,F,W,I,V, L,M,T						1° Anchor F,I,Y,W,L,M
B7	preferred	F,W,Y (3/5) L,I,V,M, (3/5)	1° Anchor P	F,W,Y (4/5)			F,W,Y, (3/5)	1° Anchor V,I,L,F,M,W,Y,A
	deleterious	D,E (3/5); P(5/5); G(4/5); A(3/5); Q,N, (3/5)			D,E, (3/5)	G, (4/5)	Q,N, (4/5)	D,E, (4/5)
B27		1° Anchor R,H,K						1° Anchor F,Y,L,W,M,Y,A
B44		1° Anchor E,D						1° Anchor F,W,Y,L,I,M,Y,A
B58		1° Anchor A,T,S						1° Anchor F,W,Y,L,I,V,M,A
B62		1° Anchor Q,L,I,V,M, P						1° Anchor F,W,Y,M,I,V,L,A

		POSITION								
		1	2	3	4	5	6	7	8	C-terminus
<u>MOTIFS</u>										
A1	preferred	G,F,Y,W,	1°Anchor S,T,M,	D,E,A,	Y,F,W,		P,	D,E,Q,N,	Y,F,W,	1°Anchor Y
9-mer										
	deleterious	D,E, "		R,H,K,L,I,V M,P,	A,	G,	A,			
<hr/>										
A1	preferred	G,R,H,K	A,S,T,C,L,I V,M,	1°Anchor D,E,A,S	G,S,T,C,		A,S,T,C,	L,I,V,M,	D,E,	1°Anchor Y
9-mer										
	deleterious	A	R,H,K,D,E, P,Y,F,W,		D,E,	P,Q,N,	R,H,K,	P,G,	G.P.	

## POSITION

	1	2	3	4	5	6	7	8	9 or C-terminus	C-terminus
A1 preferred 10-mer	Y,F,W,	<u>1°Anchor</u> S,T,M	D,E,A,Q,N,	A,	Y,F,W,Q,N,	P,A,S,T,C,	G,D,E,	P,	<u>1°Anchor</u> Y	
deleterious	G,P,		R,H,K,G,L,I V,M,	D,E,	R,H,K,	Q,N,A	R,H,K,Y,F, W,	R,H,K,	A	
A1 preferred 10-mer	Y,F,W,	S,T,C,L,I,V M,	<u>1°Anchor</u> D,E,A,S	A,	Y,F,W,	P,G,	G,	Y,F,W,	<u>1°Anchor</u> Y	
deleterious	R,H,K,	R,H,K,D,E, P,Y,F,W,			P,	G,	P,R,H,K,	Q,N,		
A2.1 preferred 9-mer	Y,F,W,	<u>1°Anchor</u> L,M,I,Y,Q, A,T	Y,F,W,	S,T,C,	Y,F,W,	A,	P	<u>1°Anchor</u> V,L,I,M,A,T		
deleterious	D,E,P,		D,E,R,K,H			R,K,H	D,E,R,K,H			
A2.1 preferred 10-mer	A,Y,F,W,	<u>1°Anchor</u> L,M,I,Y,Q, A,T	L,V,I,M,	G,		G,		F,Y,W,L, V,I,M,	<u>1°Anchor</u> V,L,I,M,A,T	
deleterious	D,E,P,		D,E,	R,K,H,A,	P,		R,K,H,	D,E,R,K, H,	R,K,H,	

POSITION									
	1	2	3	4	5	6	7	8	9 or C-terminus
A3 preferred	R,H,K,	1°Anchor L,M,V,I,S, A,T,F,C,G D	Y,F,W,	P,R,H,K,Y, F,W,	A,	Y,F,W,	P,	1°Anchor K,Y,R,H,F,A	C-terminus
deleterious	D,E,P,		D,E						
A11 preferred	A,	1°Anchor V,T,L,M,I, S,A,G,N,C, D,F	Y,F,W,	Y,F,W,	A,	Y,F,W,	Y,F,W,	1°Anchor K,,R,Y,H	
deleterious	D,E,P,						A	G,	
A24 preferred 9-mer	Y,F,W,R,H,K,	1°Anchor Y,F,W,M		S,T,C		Y,F,W,	Y,F,W,	1°Anchor F,L,I,W	
deleterious	D,E,G,		D,E,	G,	Q,N,P,	D,E,R,H,K,	G,	A,Q,N,	
A24 preferred 10-mer		1°Anchor Y,F,W,M		P,	Y,F,W,P,		P,	1°Anchor F,L,I,W	
deleterious			G,D,E	Q,N	R,H,K	D,E	A'	Q,N,	D,E,A,
A3101 preferred	R,H,K,	1°Anchor M,V,T,A,L, I,S	Y,F,W,	P,		Y,F,W,	Y,F,W,	1°Anchor R,K	
deleterious	D,E,P,		D,E,		A,D,E,	D,E,	D,E,	D,E,	

POSITION									
	1	2	3	4	5	6	7	8	9 or C-terminus 1°Anchor R,K
A3301 preferred		1°Anchor M,V,A,L,F, I,S,T	Y,F,W				A,Y,F,W		
deleterious	G,P		D,E						
A6801 preferred	Y,F,W,S,T,C,	1°Anchor A,V,T,M,S, L,I			Y,F,W,L,I, V,M		Y,F,W,	P,	1°Anchor R,K
deleterious	G,P,		D,E,G,		R,H,K,			A,	
B0702 preferred	R,H,K,F,W,Y,	1°Anchor P	R,H,K,		R,H,K,	R,H,K,	R,H,K,	P,A,	1°Anchor L,M,F,W,Y,A, I,V
deleterious	D,E,Q,N,P,		D,E,P,	D,E,	D,E,	G,D,E,	Q,N,	D,E,	
B3501 preferred	F,W,Y,L,I,V,M,	1°Anchor P	F,W,Y,				F,W,Y,		1°Anchor L,M,F,W,Y,I, V,A
deleterious	A,G,P,				G,		G,		



POSITION									
	1	2	3	4	5	6	7	8	9 : or C-terminus
B531 preferred	L,I,V,M,F,W,Y, S,T,C,	<i>1°Anchor</i> P	F,W,Y,	S,T,C,	F,W,Y,		G,	F,W,Y,	<i>1°Anchor</i> L,I,V,F,W, Y,A,M
deleterious	A,G,P,D,E,R,H,K, S,T,C,				D,E,	G,	D,E,Q,N,	G,D,E,	
B5301 preferred	L,I,V,M,F,W,Y, S,T,C,	<i>1°Anchor</i> P	F,W,Y,	S,T,C,	F,W,Y,		L,I,V,M,F, W,Y,	F,W,Y,	<i>1°Anchor</i> I,M,F,W,Y, A,L,Y
deleterious	A,G,P,Q,N,					G,	R,H,K,Q,N,	D,E,	
B5401 preferred	F,W,Y,	<i>1°Anchor</i> P	F,W,Y,L,I,V M,		L,I,V,M,		A,L,I,V,M,	F,W,Y,A,P,	<i>1°Anchor</i> A,T,I,V,L, M,F,W,Y
deleterious	G,P,Q,N,D,E,		G,D,E,S,T,C,		R,H,K,D,E,	D,E,	Q,N,D,G,E,	D,E,	

Italicized residues indicate less preferred or "tolerated" residues.  
 The information in Table II is specific for 9-mers unless otherwise specified.  
 Secondary anchor specificities are designated for each position independently.

Table III

MOTIFS	POSITION								
	1° anchor 1	2	3	4	5	1° anchor 6	7	8	9
DR4 preferred	F, M, Y, L, I, V, W	M	T		I	V, S, T, C, P, A, L, I, M	M, H		M, H
deleterious				W			R		W, D, E
DR1 preferred	M, F, L, I, V, W, Y			P, A, M, Q		V, M, A, T, S, P, L, I, C	M		A, V, M
deleterious		C	C, H	F, D	C, W, D		G, D, E	D	
DR7 preferred	M, F, L, I, V, W, Y	M	W	A		I, V, M, S, A, C, T, P, L	M		I, V
deleterious		C		G			G, R, D	N	G
DR Supermotif	M, F, L, I, V, W, Y					V, M, S, T, A, C, P, L, I			
DR3 MOTIFS	1° anchor 1	2	3	1° anchor 4	5	1° anchor 6			
motif a preferred	L, I, V, M, F, Y			D					
motif b preferred	L, I, V, M, F, A, Y			D, N, Q, E, S, T		K, R, H			

Italicized residues indicate less preferred or "tolerated" residues. Secondary anchor specificities are designated for each position independently.

Table IV: HLA Class I Standard Peptide Binding Affinity.

ALLELE	STANDARD PEPTIDE	SEQUENCE	SEQ ID NO:	STANDARD BINDING AFFINITY (nM)
A*0101	944.02	YLEPAIAKY	51487	25
A*0201	941.01	FLPSDYFPSV	51488	5.0
A*0202	941.01	FLPSDYFPSV	51488	4.3
A*0203	941.01	FLPSDYFPSV	51488	10
A*0205	941.01	FLPSDYFPSV	51488	4.3
A*0206	941.01	FLPSDYFPSV	51488	3.7
A*0207	941.01	FLPSDYFPSV	51488	23
A*6802	1072.34	YVIKVSARV	51489	8.0
A*0301	941.12	KVFPYALINK	51490	11
A*1101	940.06	AVDLYHFLK	51491	6.0
A*3101	941.12	KVFPYALINK	51490	18
A*3301	1083.02	STLPETYVRR	51492	29
A*6801	941.12	KVFPYALINK	51490	8.0
A*2402	979.02	AYIDNYNKF	51493	12
B*0702	1075.23	APRTLVL	51494	5.5
B*3501	1021.05	FPFKYAAAF	51495	7.2
B51	1021.05	FPFKYAAAF	51495	5.5
B*5301	1021.05	FPFKYAAAF	51495	9.3
B*5401	1021.05	FPFKYAAAF	51495	10

**Table V. HLA Class II Standard Peptide Binding Affinity.**

Allele	Nomenclature	Standard Peptide	Sequence	SEQ ID NO:	Binding Affinity (nM)
DRB1*0101	DR1	515.01	PKYVKQNTLKLAT	51496	5.0
DRB1*0301	DR3	829.02	YKTIAFDEEARR	51497	300
DRB1*0401	DR4w4	515.01	PKYVKQNTLKLAT	51496	45
DRB1*0404	DR4w14	717.01	YARFQSQTTLKQKT	51498	50
DRB1*0405	DR4w15	717.01	YARFQSQTTLKQKT	51498	38
DRB1*0701	DR7	553.01	QYIKANSKFIGITE	51499	25
DRB1*0802	DR8w2	553.01	QYIKANSKFIGITE	51499	49
DRB1*0803	DR8w3	553.01	QYIKANSKFIGITE	51499	1600
DRB1*0901	DR9	553.01	QYIKANSKFIGITE	51499	75
DRB1*1101	DR5w11	553.01	QYIKANSKFIGITE	51499	20
DRB1*1201	DR5w12	1200.05	EALHQLKINPYVLS	51500	298
DRB1*1302	DR6w19	650.22	QYIKANAKFIGITE	51499	3.5
DRB1*1501	DR2w2 $\beta$ 1	507.02	GRTQDENPVVHFFKNIV TPRTPPP	51501	9.1
DRB3*0101	DR52a	511	NGQIGNDPNRDIL	51502	470
DRB4*0101	DRw53	717.01	YARFQSQTTLKQKT	51498	58
DRB5*0101	DR2w2 $\beta$ 2	553.01	QYIKANSKFIGITE	51499	20

Table VI

HLA-supertype	Allele-specific HLA-supertype members	
	Verified <sup>a</sup>	Predicted <sup>b</sup>
A1	A*0101, A*2501, A*2601, A*2602, A*3201	A*0102, A*2604, A*3601, A*4301, A*8001
A2	A*0201, A*0202, A*0203, A*0204, A*0205, A*0206, A*0207, A*0209, A*0214, A*6802, A*6901	A*0208, A*0210, A*0211, A*0212, A*0213
A3	A*0301, A*1101, A*3101, A*3301, A*6801	A*0302, A*1102, A*2603, A*3302, A*3303, A*3401, A*3402, A*6601, A*6602, A*7401
A24	A*2301, A*2402, A*3001	A*2403, A*2404, A*3002, A*3003
B7	B*0702, B*0703, B*0704, B*0705, B*1508, B*3501, B*3502, B*3503, B*3503, B*3504, B*3505, B*3506, B*3507, B*3508, B*5101, B*5102, B*5103, B*5104, B*5105, B*5301, B*5401, B*5501, B*5502, B*5601, B*5602, B*6701, B*7801	B*1511, B*4201, B*5901
B27	B*1401, B*1402, B*1509, B*2702, B*2703, B*2704, B*2705, B*2706, B*3801, B*3901, B*3902, B*7301	B*2701, B*2707, B*2708, B*3802, B*3903, B*3904, B*3905, B*4801, B*4802, B*1510, B*1518, B*1503
B44	B*1801, B*1802, B*3701, B*4402, B*4403, B*4404, B*4001, B*4002, B*4006	B*4101, B*4501, B*4701, B*4901, B*5001
B58	B*5701, B*5702, B*5801, B*5802, B*1516, B*1517	
B62	B*1501, B*1502, B*1513, B*5201	B*1301, B*1302, B*1504, B*1505, B*1506, B*1507, B*1515, B*1520, B*1521, B*1512, B*1514, B*1510

- Verified alleles include alleles whose specificity has been determined by pool sequencing analysis, peptide binding assays, or by analysis of the sequences of CTL epitopes.
- Predicted alleles are alleles whose specificity is predicted on the basis of B and F pocket structure to overlap with the supertype specificity.

WHAT IS CLAIMED IS

1. An isolated prepared human papilloma virus (HPV) epitope consisting of a sequence selected from the group consisting of the sequences set out in Tables VII-XX.
2. A composition of claim 1, wherein the epitope is admixed or joined to a CTL epitope.
3. A composition of claim 2, wherein the CTL epitope is selected from the group set out in claim 1.
4. A composition of claim 1, wherein the epitope is admixed or joined to an HTL epitope.
5. A composition of claim 4, wherein the HTL epitope is selected from the group set out in claim 1.
6. A composition of claim 4, wherein the HTL epitope is a pan-DR binding molecule.
7. A composition of claim 1, comprising at least three epitopes selected from the group set out in claim 1.
8. A composition of claim 1, further comprising a liposome, wherein the epitope is on or within the liposome.
9. A composition of claim 1, wherein the epitope is joined to a lipid.
10. A composition of claim 1, wherein the epitope is joined to a linker.
11. A composition of claim 1, wherein the epitope is bound to an HLA heavy chain,  $\beta$ 2-microglobulin, and streptavidin complex, whereby a tetramer is formed.
12. A composition of claim 1, further comprising an antigen presenting cell, wherein the epitope is on or within the antigen presenting cell.
13. A composition of claim 12, wherein the epitope is bound to an HLA molecule on the antigen presenting cell, whereby when a cytotoxic lymphocyte (CTL) is present that is restricted to the HLA molecule, a receptor of the CTL binds to a complex of the HLA molecule and the epitope.

14. A clonal cytotoxic T lymphocyte (CTL), wherein the CTL is cultured *in vitro* and binds to a complex of an epitope selected from the group set out in Tables VII-XVIII, bound to an HLA molecule.

15. A peptide comprising at least a first and a second epitope, wherein the first epitope is selected from the group consisting of the sequences set out in Tables VII-XX;  
wherein the peptide comprise less than 50 contiguous amino acids that have 100% identity with a native peptide sequence.

16. A composition of claim 15, wherein the first and the second epitope are selected from the group of claim 14.

17. A composition of claim 16, further comprising a third epitope selected from the group of claim 15.

18. A composition of claim 15, wherein the peptide is a heteropolymer.

19. A composition of claim 15, wherein the peptide is a homopolymer.

20. A composition of claim 15, wherein the second epitope is a CTL epitope.

21. A composition of claim 15, wherein the second epitope is a PanDR binding molecule.

22. A composition of claim 1, wherein the first epitope is linked to an a linker sequence.

23. A vaccine composition comprising:  
a unit dose of a peptide that comprises less than 50 contiguous amino acids that have 100% identity with a native peptide sequence of HPV, the peptide comprising at least a first epitope selected from the group consisting of the sequences set out in Tables VII-XX; and;  
a pharmaceutical excipient.

24. A vaccine composition in accordance with claim 23, further comprising a second epitope.

25. A vaccine composition of claim 23, wherein the second epitope is a PanDR binding molecule.

26. A vaccine composition of claim 23, wherein the pharmaceutical excipient comprises an adjuvant.
27. An isolated nucleic acid encoding a peptide comprising an epitope consisting of a sequence selected from the group consisting of the sequences set out in Tables VII-XX.
28. An isolated nucleic acid encoding a peptide comprising at least a first and a second epitope, wherein the first epitope is selected from the group consisting of the sequences set out in Tables VII-XX; and wherein the peptide comprises less than 50 contiguous amino acids that have 100% identity with a native peptide sequence.
29. An isolated nucleic acid of claim 28, wherein the peptide comprises at least two epitopes selected from the sequences set out in Tables VII-XX.
30. An isolated nucleic acid of claim 29, wherein the peptide comprises at least three epitopes selected from the sequences set out in Tables VII-XX.
31. An isolated nucleic acid of claim 28, wherein the second peptide is a CTL epitope.
32. An isolated nucleic acid of claim 20, wherein the second peptide is an HTL epitope.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/33549

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 39/00, 39/12

US CL : 424/192.1; 204.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/192.1; 204.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WEST, BIOSIS, CAPLUS

search terms: papillomavirus, epitop? peptid?, L1, E1, HPV7, vaccine, HTL, CTL

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,855,891 A (LOWY et al) 05 January 1999, see the abstract.	1-32
Y	US 5,753,233 A (BLEUL et al) 19 May 1998, see the claims.	1-32
Y	US 5,716,620 A (LOWY et al) 10 February 1998, see the claims.	1-32
Y	US 5,871,998 A (LOWY et al) 16 February 1999, see claim 1.	1-32
Y	US 5,985,610 A (LOWY et al) 16 November 1999, see the claims.	1-32
Y	US 5,618,536 A (LOWY et al) 08 April 1997, see the claims.	1-32

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 MARCH 2001

Date of mailing of the international search report

26 APR 2001

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**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/US00/33549**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/31476 A1 (THE UNIVERSITY OF QUEENSLAND) 23 November 1995, see the claims.	1-32
A	US 5,180,806 A (DILLNER et al) 19 January 1993, see the abstract.	1-32